

STUDIES ON THE POLYSACCHARIDES OF
ICELAND MOSS (CETRARIA ISLANDICA)

by

N. B. Chanda, M.Sc.

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PART I. LICHENIN.

ANNANDALE

POLTON

I N T R O D U C T I O N .

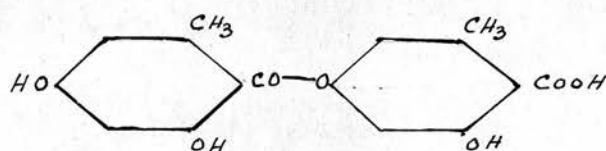
This investigation deals with the constitutions of "Lichenin" and "Isolichenin," obtained from "Cetraria Islandica," which is commonly known as "Iceland Moss." Isolichenin will be dealt with in the second part of the thesis. Iceland moss belongs to the peculiar group of plants known as "Lichens." Lichens are plants consisting of light-coloured hyphal filaments and cells or groups of cells of a green, blue-green, or more rarely brownish or reddish colour. The green bodies and hyphal filaments are in close relationship with each other and contribute to the formation of a quite constant structure, the thallus, which, in form, varies according to habitat. Lichens are found on a wide variety of substrata, from the High Arctic to the Tropics and from mountain top to seashore, contributing richly, in some cases, to the flora of a region. The total number of known species is over 15,500 comprising 60 families and over 400 genera (Catalogus lichenum Universalis, 1932, 8, [11-20]: 161-320. Borntraeger: Leipzig). A few of the important lichens are named below:

Cetraria islandica
Evernia prunastri
Umbilicaria pustulata
Cladonia alpestris
Evernia vulpina
Umbilicaria hirsuta
etc.

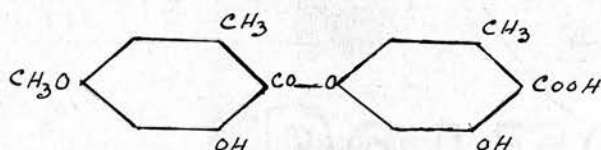
In the past 60 years about 200 compounds have been

extracted from various species of lichens. These compounds have not been found in any other group of plants and are thought to be due to the peculiar morphological association of these plants. The chemical constitution of about 20 of these compounds is known, although the empirical formula of most of them have been published. With few exceptions, all contain oxygen and are acid or neutral. A few important lichen acids (Depside) are named below with their constitutional formulae:

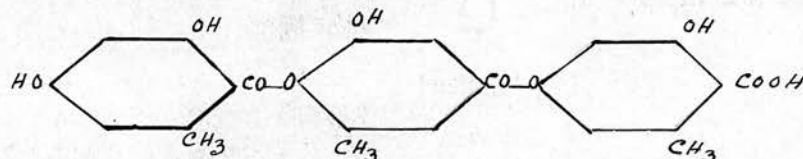
Lecanoric acid (p-di-orsellinic acid)



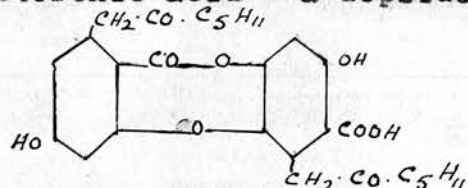
Evernic acid (p-monomethyl-lecanoric acid)



Gyrophoric acid - a naturally occurring tridepside.



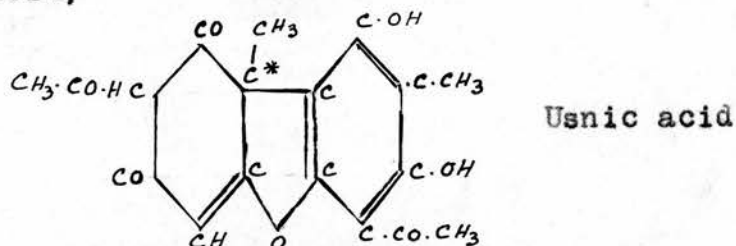
Alectoronic acid - a depsidone.



It may be noted here that lichens are the only natural

source of "depsides" and "depsidones."

Recent investigations have shown that crude extracts of many lichens (*Everina furfuracea*, *Lobaria pulmonaria*, *Cladonia borbonica*, *Ramalina reticulata*, etc.), and some of the individual lichen acids, such as Usnic acid,



have considerable antibacterial activity, including activity against the tubercle bacillus. Experiments with animals indicate that some of these substances may prove suitable for Chemotherapy, particularly for combating infections resistant to currently used drugs. These interesting discoveries give some justification for the traditional use of lichens, in many different countries, for treating certain pulmonary affections. Vartia (Ann.Med.Exper.Biol.Fenniae, 27, 1, 46, 1949) states that in Finland hot aqueous extract of reindeer-moss (*Cladonia alpestris*, *Cladonia rangiferina* or *Cladonia sylvatica*) is traditionally used as a remedy for tuberculosis. Of 82 species tested, Usnic acid was found in 22. These 22 species were all active against *Sarcina aurea*, *Staph.aureus*, *Step.pyogenes*, *B.subtilis*, and *B.megatherium*; the majority were also active against *C.diphtheriae*. In a survey of the antibacterial activity of *Cetraria islandica*, Vartia found it to be

active against *Sarcina aurea*, *Staph.aureus*, *Strep. pyogenes*, *E.coli*, *P.vulgaris*, and *B.megatherium*. In 1947 Stoll and his collaborators (*Experientia*, 3, 3, 115, 1947) isolated dl-usnic acid from *Cetraria islandica*, the yield being 0.04 per cent. They showed that its antibacterial activity is independent of its optical activity. All forms of usnic acid inhibited strains of *M.tuberculosis hominis* and *M.tuberculosis bovis* at dilutions ranging from 1:500,000 to 1:800,000.

As early as 1808, Berzelius (*Ann.Chem.*, 90, 277, 1814; *Bull,Pharm.* 6: 536-550) discovered that many species of lichens on extraction with hot water yielded a gelatinizing substance which he named "Flechten stärke" (Lichenin); later investigations have shown this to be not a single substance but a number of related carbohydrates. The names of some of these substances are indicative of the species from which they have been extracted, as "evernin" from *Evernia prunastri* and "Usnici" from *Usnea barbata*. Of late years, Karrer and his collaborators have characterized lichenin as the "reserve cellulose" of lichens and found it in the seeds of many higher plants. Lichen starch (lichenin) is said by Haas and Hill to be chemically, but not physically, identical with the amylose of starch grains.

The employment of lichens as raw materials in pastries, confectionery, food, and in the production of

alcohol depends largely on the properties of lichenin. The presence of a certain number of phenols, acid-phenols, and acid-phenol-ethers together with other substances in extracts of "Evernia prunastri" and other lichens, forms the basis of their use in perfumery and cosmetics. The tinctorial properties of lichens, long used in dye industries, are for the most part ^{due to} derivatives of orcinols in the species of "Roccella."

The particular lichen, with which this investigation is concerned is Cetraria islandica. The habitat of Iceland moss, in particular, is Iceland, but it is also found in the Scandinavian countries. Like most of the other members of its class it contains lichenin to the extent of 50-60% of its dry weight. Iceland moss probably rates first as human lichen food. Preparation for this purpose includes removal of foreign material and soaking; then it is dried and powdered. It is made ready for human consumption by boiling as a broth which on cooling sets as a jelly. Milk is added and in this form the lichen is the basis of various light and easily digested soups and other delicacies said to be of high value for dyspeptics. It may be made into bread, porridge or gruel. Schneider says of this "moss," "Inhabitants of Iceland, Norway, Sweden mix this with various cereals and mashed potatoes from which an

uncommonly healthful bread was prepared." Lynge (Smith - Brit.Mycol.soc.Trans., 6, 17,31, 1918) quotes a tradition "that there was no starvation at Modun in 1812 as long as there was bread-moss (~~bro~~dmose) left in the forest."

Icelanders feed *Cetraria islandica* to their cattle and ponies. It has also been reported good for oxen (Willemet - Lichenographie economique ou histoire des lichens utiles, 1787), while the richness of the milk of the small cows of ~~the~~ northern Scandinavia is attributed to this food (Johnson, C.P. - The useful plants of Great Britain, 1861).

Iceland moss was given an important place in medicine by Linnaeus in 1737. It has been used in chronic affections as an emollient and tonic and it would indeed have been a "Divine gift to man" had it lived up to all its prescriptions.

It is the only lichen included in the British Pharmacopeia. Today, it is used as a substitute for salve bases for the preparation of emulsions, the reduction of the bitter taste in certain drugs, as a laxative and as a culture medium for bacteria (Piorkowski - Ber.Pharm.Ges., 26, 192-193, 1916).

The chemical composition of Iceland moss has been investigated by several workers. A detailed analysis was carried out by Berzelius (Lehrbuch der Chemie - Vol.7, page 446), and the details are given below,

copied word for word from his own hand book of chemistry:

	Per cent.
Greenwax	1.6
Cetrarin	3.0
Uncrystallisable sugar	3.6
Gum	3.7
Extractabsatz	7.0
Moos starch	44.6
Potassium bilichenate, lichensaure. Kalkerde, and phosphorsaurer kalk	1.9
Starkeartiges skelett	36.2
(Gewichtszuschuss	1.6)

Subsequent investigations (E. Poulsson, et.al., - Pharmakologischen Institute der Universitat Christiania) yielded similar results for the composition of the moss.

Iceland moss thus contains not less than some 80% of carbohydrates, partly soluble and partly insoluble in hot water. The most important of the water-soluble carbohydrates is that called by Berzelius "Moosstärke," and now commonly called "Lichenin." It is obtained from the lichen, by boiling it with water, as a stiff mucus, which sets on cooling to a jelly, used in medicine as "Gelatina Lichenis islandici." In the moist state this jelly gives a blue colour with iodine which depends, however, as shown by Berg

(Bekurts Jahresberichte, 1873, page 19), not on lichenin itself, but on Isolichenin, which accompanies the former in very small quantities. Isolichenin is very soluble in water and is found essentially in the liquid poured off from the jelly. Isolichenin, according to König and Schubert (Mont., 8, 452, 1887), differs from lichenin by being destroyed by the action of malt extract at 60°C. Its aqueous solution is strongly d-rotatory, while lichenin has a sp. rotation of below 10° in caustic soda solution.

Besides lichenin and isolichenin, other polysaccharides present in the cell-wall of Iceland moss have also been studied. Granichsteden and Percival, (J.C.S., 54-58, 1943) found the so-called "hemicellulose" of the moss to give on hydrolysis a mixture of sugars containing glucose (89%); galactose (8%), mannose (3%) and a uronic acid (5%), probably D-glucuronic acid. Hydrolysis of the methylated "hemicellulose" was found to give four different trimethyl sugars: 2,3,4-trimethyl glucose; 2,3,6-trimethyl glucose; 2,4,6-trimethyl glucose and 3,4,6-trimethyl glucose besides the end group. This fact suggests that the hemicellulose is either a mixture of at least four polysaccharides differing in the type of linkage between the glucose residues (1:2-, 1:3-, 1:4-, 1:6-) or that it is a mixture of polysaccharides in each of which mixed linkages are present. The complex

character of the hemicellulose is quite evident from these results.

Besides lichens, polysaccharides resembling lichenin also occur (Karrer and Staub, - *Helv.*, 7, 159, 1924) in many other plants, such as barley, oats, maize, spinach, beans, hyacinth bulbs, etc.

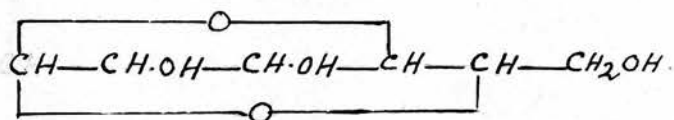
Lichenin, considered to be a modified form of cellulose, was found to give a quantitative yield of glucose by enzymatic hydrolysis. The apparent similarity of the building unit in cellulose and lichenin was established by the isolation of cellobiose octa-acetate (Karrer, Joos and Staub, - *Helv.*, 6, 800-816, 1923) by means of acetolysis and by the isolation of 2,3,6-trimethyl glucose in yields similar to those obtained from cellulose by methylation and hydrolysis (P.Karrer and K. Nishida, - *Helv.*, 7, 363, 1924). Although both polysaccharides, therefore, appear to be made up of 1,4- β -glucosido glucose units (Haworth, *Chem. and Ind.*, 925, 1939), they differ sharply in their physical properties in that lichenin easily forms colloidal solution in hot water from which it can be precipitated by alcohol and is less resistant to hydrolytic agents and enzymes. The derivatives of cellulose and lichenin differ in their optical rotations and also in their X-ray patterns - these results point to a difference in the macro-molecular structure of the two polysaccharides. On the other hand,

evidence as to the similarity of their basic structures was presented by Otto (Helv., 9, 31-32, 1926), who observed identical X-ray patterns both with modified cellulose (oxycellulose and hydrocellulose) and lichenin, showing that the crystalline constituents of these substances are identical. Karrer (Z.Biochem., 136, 537-541, 1923) claimed the preparation of lichenin acetate which was similar to cellulose acetate in having a similar rotation in chloroform, $[\alpha]_D^{19} = -23.8^\circ$, and that both are practically non-reducing and yield sodium hydroxide addition compounds of the same composition. From these facts, he suggested that cellulose and lichenin are closely allied substances. Pringsheim (Z.Physiol.Chem., 137, 265-271, 1924) also claimed the hydrolysis of lichenin (at 37°C.; pH 5; 4 days) to cellobiose by means of "lichenase," an enzyme present in the alimentary canal of edible snails.

Due to this close relationship with cellulose, the problem of lichenin structure has kept a number of investigators busy for the last 30 years, in the hope that its clarification would go a long way to the understanding of the make-up of the cellulose molecule and those of the other allied polysaccharides.

In the year 1925, Pringsheim and his co-workers (Ber., 58, 2135-2143, 1925) obtained on glycerolysis of lichenin a non-crystalline cold water soluble substance, which they named "Lichosan," considering it to

be a glucose anhydride.



This suggestion was made after the determination of the molecular weight of its acetyl derivative by the cryoscopic method. They observed that in water it rapidly polymerises to lichenin, which according to the X-ray pattern, was completely identical with the natural material. It, therefore, appeared probable that lichosan was the unit structure of lichenin, in which the units are linked together only by subsidiary valencies. The optical inactivity of lichenin (according to Pringsheim) and lichosan in water was remarkable and apparently due to the compensation of the activity of the aldehydic C-atom by that of the four remaining asymmetric C-atoms. By comparing these results with those obtained with cellulose, they hoped to solve the question^{of} whether the two polysaccharides made up of possibly identical building units can differ as much as do lichenin and cellulose mainly due to the different use of the residual valencies of the participating basic units.

Pringsheim made use of the cryoscopic method to investigate his lichosan and from this point of view, the work of Hess and Schultze (Ann., 448, 99-120, 1926) is worth considering here. Hess obtained results from cellulose in copper tetramine solutions from which it

follows that cellulose behaves in these solutions as if it consisted of chemically independent molecules of the size $C_6H_{10}O_5$. On regeneration from such solution, cellulose again showed the property of being insoluble in neutral solvents. Its chemical properties, as well as those of its acetyl and methyl derivatives, were also identical with the original substance. These observations led him to suggest that the cellulose structure embodies a quantitative rather than a fundamental difference to that of the water soluble and easily crystallisable carbohydrates. Cryoscopic values for cellulose acetate in glacial acetic acid were found to vary, which occasionally indicated an even lower molecular weight than that of a basic $C_6H_{10}O_5$ unit. The reason for it was found to be due to air absorption by the solution and elimination of air gave results in agreement with one anhydro glucose unit. However, even with the exclusion of air, after several days of constant molecular weight, reaggregation caused decrease of depression, indicating increase of molecular weight. He concluded, therefore, that cryoscopic molecular weight values of polysaccharides which point to multiples of $C_6H_{10}O_5$ molecules give no information as to the structure and he pointed out that "there is no need to give different names to the high polymer substance and the non-molecularly dispersed particles derived from it." He also investigated

whether such drastic action as glycerolysis need be used to get cryoscopic results from lichenin corresponding to one anhydro-glucose. According to Hess, lichenin and cellulose are different because their acetates differ in rotations, though the behaviour of the lichenin acetate solution during cryoscopic experiments in the presence of air was identical with that of cellulose. His general conclusions as to the nature of the polysaccharide are the same as those expressed on cellulose. As regards the solubility of lichosan in water, he suggested that it was due to the presence of decomposition products from the glycerolysis process.

Bergmann (Ann., 452, 151-158, 1927), following up Pringsheim's glycerolysis experiment on lichenin, prepared a hexosan, which had different properties from those ascribed to lichosan and, therefore, he named it as "Licho-hexosan." The pure hexosan exhibited no activity in a 2.5% aqueous solution at 20°C., but at 70°C., when, according to Pringsheim, dissociation is complete ($M = 162$), it has $[\alpha]_D^{20} = +8.7$ (constant). Hence the optical inactivity cannot be due to the compensation of the activity of the aldehydic C-atoms by that of the four remaining C-atoms (as assumed by Pringsheim) but it must be due to association. He also rejected the idea that lichosan is the unit member of the lichenin molecule.

Hess (Ann., 455, 180, 1927) summarises his view on the subject by saying that Bergmann's licho-hexosan was nothing more than impure degraded lichenin and that Pringsheim had never been able to give any positive proof as to the uniformity of his products. He also emphasized the action of the solvent: in low concentrations molecular dispersion takes place by breaking up the forces between the molecules in the same way as happens with all other substances, with one difference, ^{viz.,} that a great tendency for reaggregation exists.

A few years later, Berner (Ann., 500, 52-61, 1932) stepped into this field and carried out some experiments on the action of glycerol at 240°C. on lichenin in a nitrogen atmosphere. From the results, he came to two conclusions: (a) a fraction of lichenin is not degraded (or slightly) but merely dissolved by glycerine, which is in turn absorbed, causing increased water solubility, and (b) a fraction of lichenin is degraded to cold water soluble, non-reducing fragments (lichosan, licho-hexosan, etc.), which in turn fix the glycerine chemically. Analysis of the lichosan acetate supported this view.

The work of Hess, Pringsheim et al., on lichenin (discussed above) and on other polysaccharides now possesses, in general, only historical interest as a result of the new concept of carbohydrate chemistry

introduced by Haworth and his school since 1927. Hess and Pringsheim's work was carried out in the period between 1920-30, when the polysaccharides were believed to be formed by a molecular association of small units of definite constitution joined together by residual or auxiliary valencies and that when the associated product or polymer was subjected to a variety of external conditions it might revert to the original units. According to this idea, starch or cellulose was considered to be a polymer of diglucose anhydride or triglucose anhydride. Such concepts of polysaccharide structure were contested all through by the Haworth school and also by Staudinger and Freudenberg. The allocation of the ring structure (pyranose and furanose) to sugars together with various experimental evidence bearing on the constitution of the polysaccharides led to the development of the macro-molecular theory of polysaccharide structure accepted today. According to this theory, the individual monosaccharides are conceived to be linked one to another in the polysaccharide molecule in the same way as in glycosides, forming chains of different length. Although this view appears to give a correct picture of the structure of substances of high molecular weight on the whole, it is still not certain (a) whether there is the same type of linkage between the sugar residues throughout

the glycosidic chain, (b) how long the molecules are, and (c) what is the structural arrangement of the terminal members of the chains.

Staudinger approached the problem of the nature of the lichenin molecule from the "viscosity" point of view. He was able to show (Ber., 71, 2522, 1938) that lichenin is a macromolecule such as cellulose, starch, etc., but he believed that the greater solubility of lichenin in comparison with cellulose tends to show that it is composed of molecules in string form, which are curved in the manner pictured in the ramified structure of starch. He estimated the minimum molecular weight of ^a lichenin molecule to be of the order of 10,000. In a subsequent paper (Staudinger and Lantzsch, J.prakt.Chem., 156, 65-94, 1940), Staudinger said that the lichenin from Iceland moss (prepared by the method of Karrer), treated in a number of ways, yielded two lichenins: lichenin I and lichenin II. These lichenins, as well as their acetates, exhibited different rotatory powers. By osmotic pressure measurements, the lichenins and their acetates showed the same degree of polymerization. If the acetates are saponified the products show the same rotation as the original lichenin. The molecular weights (obtained from osmotic pressure and viscosity measurements) of lichenin I and lichenin II and also that of their acetates and nitrates were found to vary

little with solvent. From all these observations, they concluded that lichenin is not colloidal, but a polymerized molecule, shorter than the cellulose molecule and with differences in the orientation of the building units.

Carter and Record (J.C.S., 664, 1939) calculated the molecular weights of various samples of methylated and acetylated lichenin from the osmotic pressure measurements. Methylated lichenin prepared from the acetate have molecular weights of about 10,700 - 13,900, corresponding to a D.P. of 66 to 86 glucose units. Acetylated and directly methylated lichenin gave values of the order of 33,000 - 37,000. The end-group assay method pointed to a minimum D.P. of about 80. All these measurements have been carried out at various concentrations because in the case of long chain molecules deviations from the Vant Hoff's law occur. π/c increases with increasing C, which necessitates extrapolation to zero concentration where the ideal condition may be assumed to hold.

In 1940, Hess and Lauridsen (Ber., 73B, 115-126, 1940) studied the constitution of lichenin by the methylation technique. They prepared the fully methylated lichenin ($-OCH_3$, 45.5%) by the methods of Haworth and Purdie and then hydrolysed the fully methylated material to the glycosides with methanolic HCl. A chemical method was used to effect the

complete separation of the end-group (tetramethyl methyl glycoside) from the other glycosides. The latter was esterified to yield phosphoric esters, the barium salts of which are insoluble in ether and petrol ether, which are good solvents for tetramethyl methyl-glycosides. This method gave results corresponding to a D.P. of 114 - 116, which is almost double the values recorded by Staudinger and Carter.

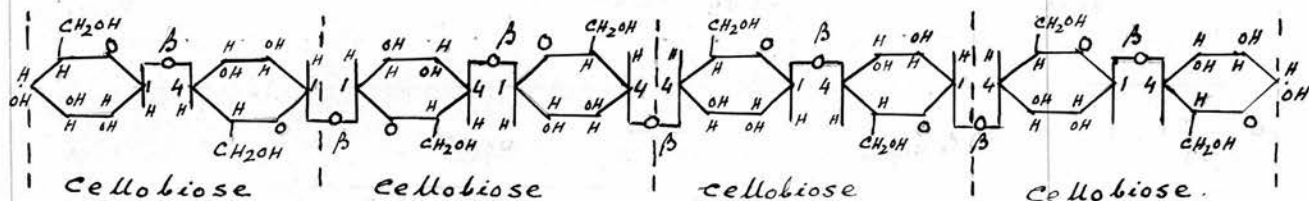
The possibility of branching in the lichenin molecule as suggested for starch (Hirst and Young, J.C.S., 1471, 1939) did not appear to arise here because the authors claimed the absence of a dimethyl glucose fraction.

The acetyl and methyl derivatives of cellulose and lichenin differ in their rotations:

Acetyl lichenin:	$[\alpha]_D^{20} = -40.3^\circ$	in CHCl_3
Acetyl cellulose:	$[\alpha]_D^{20} = -22^\circ$	in "
Methyl lichenin:	$[\alpha]_D^{20} = -13.7^\circ$	in "
Methyl cellulose:	$[\alpha]_D^{20} = -4.7^\circ$	in "

These values were quoted by Hess and Lauridsen.

This difference according to Hess is due to a chemical difference between cellulose and lichenin. Hess tried to account^{for} the difference in properties between cellulose and lichenin by assuming a mixed linkage in the lichenin molecule. He suggested that the glucose units are linked not only in β -1,4, but also in the β -1,1 and β -4,4 positions.



This structure will explain the quantitative hydrolysis of lichenin to cellobiose (claimed by Pringsheim, Karrer, et.al.), if it is assumed that the β -1,1 and β -4,4 linkages are broken before the β -1,4-linkage. At this point, the relative stability of the three linkages, 1,4-, 4,4-, and 1,1- must be considered. The behaviour of β , β -isotrehalose towards yeast^{and} emulsin supports the 1,1-linkage splitting and it is not unlikely that the splitting of the 1,1-linkage is easier than that of the 1,4-linkage. Nothing is known about the cleavage of a 4,4-linkage and, being a true ether linkage, its splitting will probably be more difficult than that of a 1,4-linkage. Hence the formula of Hess must be taken with some reserve.

From a study of viscosity, he then compares the degree of polymerisation of lichenin with that of starch which he considers to be 52 and tries to show, on the assumption of an unbranched chain for lichenin, a relation between solubility and chain length of cellulose, starch and lichenin. Starch, he says, with

a shorter chain, is less soluble than the longer chain lichenin and this he considers to be due to the branching of the starch chain. Haworth (Chem. and Ind., 925, 1939) also once suggested that the solubility difference of lichenin and cellulose is due to many cross linkages in cellulose.

An interesting point in the nature of the lichenin molecule emerges from the work of Schmidt and his co-workers (Naturwissenschaften, 22, 172, 1934). They claimed the presence of a carboxyl group in the lichenin molecule, an observation which the above workers also claimed with cellulose. They showed that the lichenin molecule has 0.563% (calculated as CO₂ by conductimetric titration) of carbonyl group, which accounts for the presence of 47 glucose units in the chain. In this connection, their method of lichenin extraction must be noted: after treating the moss six times with chlorine dioxide and sodium sulphite at pH 6.8, the lichenin was extracted by an ammoniacal copper hydroxide solution and from the acidified solution lichenin was precipitated with alcohol. The above treatments with chlorine dioxide may be the factor responsible for the oxidation of the terminal reducing end-group to a carboxyl group.

In 1942, Drake (Biochem.Z., 313, 388-399, 1942) hydrolysed lichenin both by means of 51% H₂SO₄ and by an enzyme, Luizym, and studied the rate of hydrolysis

in the two cases, both polarimetrically and titrimetrically (estimating the amount of glucose formed). α , the titrimetric degree of splitting, was always found to be less than α' (polarimetric degree of splitting), a phenomenon also found with cellulose. The velocity of hydrolysis of lichenin was also found to be half of that of starch and double ~~of~~ that of cellulose. From these results and considering other factors like heat of activation, molecular rotation per unit, steric factor, etc., Drake put forward the view that lichenin is a β -1,4-polyglucan. Pustulin, a new polysaccharide from the lichen *Umbilicaria pustulata* ($[\alpha]_D^{20} = -44^\circ$ in water; triacetate, $[\alpha]_D^{20} = +9^\circ$ in CHCl_3) was similarly studied by Drake who suggested it to be a 1,6-polyglucan.

Recently, Meyer and Gurtler (Helv., 301, 751, 1947) studied the structure of lichenin by the periodic acid technique and also by the methylation method. They showed the presence of about 30% 1,3-linkages in the lichenin molecule, the remaining 70% being 1,4-linkages. Confirmatory evidence has been given by the reduction of the trimethyl glucoses (from the hydrolysis of the fully methylated lichenin) to the corresponding glucitols and the chromatographic (adsorption) separation of the resulting coloured compounds, 1,4,5-triazoyl 2,3,6-trimethyl glucitol and 1,3,5-triazoyl 2,4,6-trimethyl glucitol. This work

of Meyer for the first time raised the question of mixed linkages in the lichenin molecule and this is one of the main points in which lichenin structure differs from cellulose.

From the above review of the work on lichenin so far reported, it is quite evident that the problem still remains to be solved in connection with the structure of lichenin in spite of several attempts in this direction during the last 30 years. Assuming the validity of Meyer's conclusion of the presence of mixed linkages, an elucidation of the mode of arrangement of the glucose residues linked through 1,4 and 1,3-linkages within the molecule will go a long way to solve the problem of lichenin structure. This work was, therefore, undertaken to find the correct answer to the lichenin structure with the help of chromatography, a new finer technique which has proved of great value in many branches of chemistry.

It is of interest to note here the properties of Floridean polysaccharide. Floridean starch, which is known to occur in many red algae, is composed of glucose units only and periodate oxidation has shown that its molecule contains 40% 1,3-linkages, (Hirst, Jones and co-workers, J.C.S., 1468, 1949). It gives a colour with iodine from reddish-brown to violet according to the concentration of the reagents and has $[\alpha]_D^{15^\circ} +156$ in water. It is possible that the Floridean

starch bears a relation to starch similar in type to that which lichenin does to cellulose.

One of the main difficulties in carbohydrate chemistry has been the identification, separation and estimation of different sugars. The classical methods of estimation by selective precipitation, polarimetry and reduction equations or compound formation (Browne, J.A.C.S., 28, 439, 1906; Zerban and Wiley, Ind.Eng.Chem.Anal.Ed., 6, 354, 1934) are not always helpful and require large amounts of substance. This problem is further complicated when one sugar is present in a very small amount as an impurity. A new technique was, therefore, sought in order to make accurate quantitative estimation of free and methylated sugars on the micro-scale. Partition chromatography technique, developed within the last 4-5 years, affords such a method for the identification (Partridge, Nature, 158, 270, 1946) and estimation (Flood, Hirst and Jones, Nature, 160, 86, 1947; Flood, Hirst, Jones, J.C.S., 1679, 1948; Hirst, Hough and Jones, J.C.S., 928, 1949) of sugars on filter paper in minute amounts and quantitative separation on a macro-scale on a cellulose column (Hough, Jones and Wadman, Nature, 162, 448, 1948). These methods have made possible a new approach to the problem of elucidating the mystery underlying the chemistry of polysaccharides.

ANNANDALE

EXPERIMENTAL.

Extraction and Purification of Lichenin.

The solubility of lichenin in hot water was utilized in its extraction from Iceland moss. The moss (500 gm., previously well cleaned and cut into pieces) was first extracted with benzene and methanol in turn in a soxhlet apparatus to remove the waxy and colouring materials, and then powdered in a mill. The powdered moss was subjected to an overnight treatment with a cold 2% sodium carbonate solution (1.5 l.) to remove the lichen acids (Everic, Lecanoric, Gyrophoric acids, etc.), and then washed free from alkali. The neutral moss residue was extracted with a sufficient quantity of boiling water (3 l.) for 2-3 hours, the whole mixture being continuously stirred. It was filtered through a filtering cloth and the hot brown liquor containing lichenin and isolichenin was allowed to cool slowly.

The lichenin separated as a flocculent gelatinous precipitate (80%) leaving the remaining 20% and the isolichenin in solution. The lichenin was then separated by centrifuging and the mother-liquor preserved for the isolation of isolichenin.

The main impurity of lichenin is isolichenin whose presence can be detected by the development of a blue colour with iodine solution. Isolichenin is soluble in cold water. This property has been of service in removing the polysaccharide from lichenin. Lichenin was dissolved in hot water (1 l.) and allowed to cool

slowly when the lichenin separated leaving the isolichenin in solution. This process of dissolving in hot water and subsequent cooling was repeated several times until the lichenin gave no blue colour with iodine. The final purification was effected with the help of Fehling's solution in the following way.

Lichenin was dissolved in dilute sodium hydroxide solution and the solution centrifuged to remove the insoluble residue. The alkaline solution was treated with an equal volume of freshly prepared Fehling's solution when a lichenin copper complex precipitated. The copper-complex was separated by centrifuging and thoroughly dispersed in water (1 L.) by shaking for 5-6 hours. The dispersed lichenin-copper complex was decomposed with dilute acetic acid and filtered to give a clear polysaccharide solution. By adding acetone to this solution, lichenin was precipitated as a white flocculent solid. The polysaccharide was recovered by centrifuging and washed several times with 60% acetone to remove acid and then with acetone, alcohol and ether in turn to remove water. It was dried over phosphorus pentoxide in a vacuum desiccator and obtained in a white powdery state. Yield: 3 gms.

The polysaccharide was shaken with water (1 L.) for 5-6 hours to remove any degraded water soluble fractions arising from the various treatments in the

process of purification. After this extraction, it was recovered in a dry powdery state in the normal way. It may be mentioned here that the process of "incomplete precipitation" was followed both in the precipitation of the copper-complex from the alkaline solution and the free polysaccharide from the acid solution.

Qualitative Chromatographic Examination of Lichenin.

A small amount of pure lichenin (20 mg.) was hydrolysed in a sealed tube with N-sulphuric acid (2 c.c.) for 3 hours. The hydrolysate was neutralised with barium carbonate, centrifuged and the clear solution treated with Amberlite resins to remove inorganic salts and then concentrated in vacuo to a syrup. Paper chromatographic examination of the syrup showed the presence of glucose only. A crude preparation of lichenin, examined similarly, showed the presence of mannose, galactose and a pentose sugar in small quantities in addition to glucose. This result proved conclusively that lichenin was made up of glucose units only.

General properties of lichenin.

Lichenin, purified by the method previously described, was obtained in a white powdery state, soluble in alkali but insoluble in acid and cold water. Hot water dissolved the lichenin giving a moderately

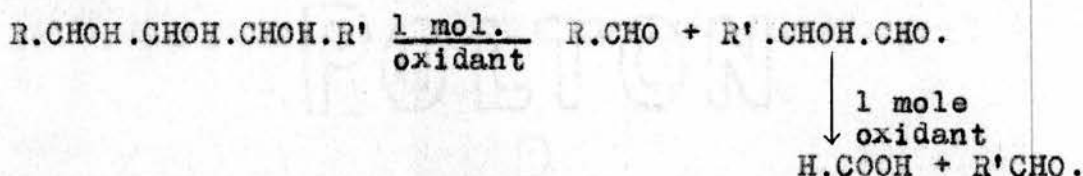
viscous solution which on cooling, deposited the lichenin as a flocculent gelatinous precipitate. The polysaccharide was reducing to Fehling's solution and different samples had sp. rotations varying from $+ 8^{\circ}$ to $+ 10^{\circ}$ in N-sodium hydroxide solutions (c. 1). Ash content: 0.88%.

Hydrolysis and quantitative estimation of glucose.

0.2095 gm. of lichenin was hydrolysed with N-sulphuric acid (5 c.c.) in a sealed tube for 3 hours. The glucose content of the hydrolysed solution by polarimetric determination was found to be 93.7%. The method of quantitative paper chromatography (Flood, Hirst and Jones, J.C.S., 1679, 1948) gave a value of 94.24%.

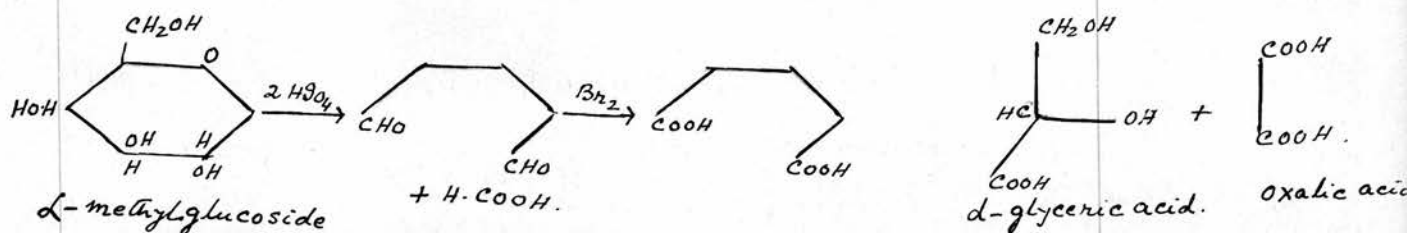
Periodate oxidation.

Periodic acid in aqueous solution at room temperature (also lead tetra acetate in glacial acetic acid solution) splits oxidatively C-C bonds where either two hydroxyl (Malaprade, Bull.Soc.Chim., 1, 833, 1934) or one hydroxyl and one amino groups (Nicolet and Shinp, J.A.C.S., 61, 1615, 1939) are attached to two adjacent carbon atoms. In the latter case ammonia is liberated. One atom of oxygen is taken up for each bond split and formic acid is formed if more than two adjacent hydroxyl groups are present.



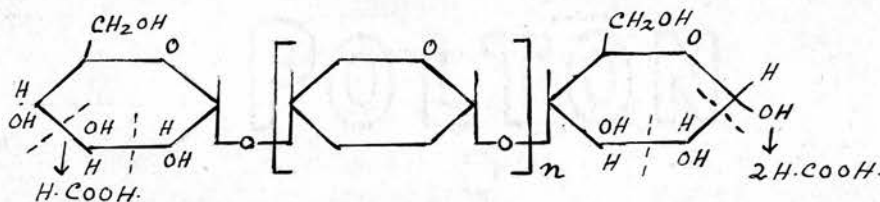
If the substance examined is not cleaved by periodic acid, it is evident that no adjacent hydroxyl groups are available.

Neuberger (J.C.S., 50, 1941) made use of periodic acid as a tool in the investigation of carbohydrate structure during the preparation of reference compounds from glucosamine. Jackson and Hudson (J.A.C.S., 58, 378, 1936; 59, 994, 1937) utilised this reaction to prove the presence of these adjacent hydroxyl groups, and hence the presence of a pyranose ring in α - and



β -methylhexopyranosides. They isolated pure d-glycolic and oxalic acids to confirm the presence of pyranose ring in the molecule.

The above consideration shows that polysaccharides built up on the pattern of starch, cellulose glycogen with chains of 1:4-linked hexopyranose residues should yield one mole of formic acid from the non-reducing end and another two moles from the reducing end:

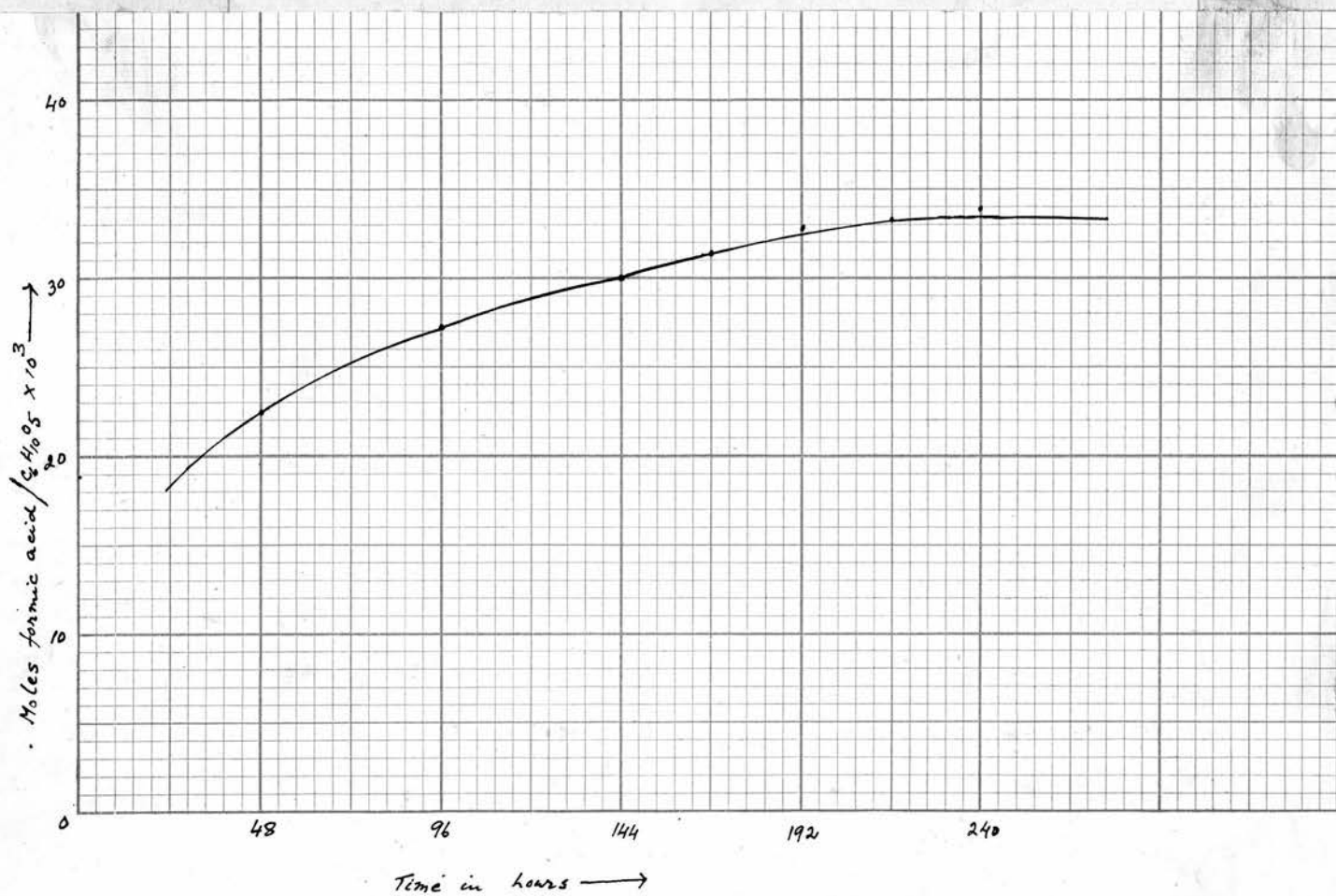


On the other hand, polysaccharides containing 1:6-linkages will give rise to one mole of formic acid for every residue which contains no other substituents. Thus, from the amounts of formic acid produced on periodate oxidation, it appears possible that considerable information (mode of linkage, ~~linkage~~, chain length, etc.) about the nature of a polysaccharide molecule would be obtained. The marked tendency for oxidation of polysaccharides by periodic acid to proceed beyond the stage depicted above (Davidson, J. Textile Inst., 32, T, 109, 1941) has hitherto restricted

the usefulness of the method until Hirst and his co-workers (J.C.S., Nat., 156, 785, 1945; J.C.S., 27, 1948) have developed a satisfactory method whereby the formic acid can be estimated with the minimum amount of over-oxidation. By this method, the polysaccharide is oxidised in an aqueous solution by a suspension of potassium periodate. As this salt is sparingly soluble in water, there is insufficient periodate present in solution to permit any significant over-oxidation. The formic acid is estimated by titration with 0.01N alkali after destroying the excess periodate with ethylene glycol. The latter reduces the excess periodate to neutral iodate and is, itself, oxidised to formaldehyde which is also neutral.

The above discussion gives us an idea of the importance of periodic acid oxidation for structural studies in carbohydrate chemistry.

Oxidation of Lichenin: The oxidation of lichenin was carried out with potassium periodate (K_2O_4) and the procedure is described briefly. A known weight of lichenin (60-70 mg.) was weighed out in a number of clean well-stoppered bottles (25 c.c.) and treated with potassium periodate (approximately four molar proportions) followed by 15 c.c. water. A few blanks were also started. The bottles were shaken continuously in the dark. Each bottle was then taken out at intervals



and the contents centrifuged to get a clear solution. 10 c.c. of the solution was then treated with 1 c.c. of ethylene glycol to destroy the periodate in solution and titrated with N/100 caustic soda, using methyl red as indicator. The number of moles of formic acid liberated per gm. mole of anhydroglucose was found to be as follows:

Time in hours.	Moles formic acid/ $C_6H_{10}O_5$ $\times 10^3$
48	22.5
96	27.1
144	30.0
168	31.4
192	32.9
216	33.2
240	33.9

The amount of formic acid liberated was not constant even at the end of 240 hours, although the increase in the day to day yield was very small. This increase may be due to over-oxidation and experimental error. If an average of the result during the period 192 to 240 hours is considered, the yield of formic acid per residue amounts to 0.0333 moles. Assuming lichenin to be a straight chain polymer, 3 moles of formic acid will be liberated per molecule of the polysaccharide. Therefore, these figures indicate that the chain length of lichenin is approximately 90 units.

Periodate uptake of Lichenin.

The amount of periodic acid consumed by a polysaccharide gives us information about the nature of the linkage present in the molecule. A periodate uptake ^{of} less than one per anhydro residue indicates the presence of some 1,3-linkages in the molecule and this type of behaviour was found with lichenin.

Approximately M/4-sodium metaperiodate solution was used in determining the periodate uptake of lichenin and the experiment was carried out in different batches to avoid the difficulties associated with insoluble polysaccharides. A known weight of dry lichenin (60-70 mg.) was weighed out in a number of clean well-stoppered bottles (25 c.c.) and treated with 15 c.c. of the periodate solutions and then shaken continuously in the dark. Each sample was taken out at certain intervals and centrifuged. The amount of periodate consumed by lichenin was then estimated by titrating 10 c.c. of the clear solution by the method of Fleury and Lange (J.Pharm.Chim., [8] 17, 107, 1933) which involved the following principle. The periodate solution containing sodium bicarbonate was allowed to react with potassium iodide in the presence of excess of standard sodium arsenite solution. The periodate was quantitatively reduced to iodate and the iodine formed on oxidation of the iodide reacted with arsenite. When the reduction was complete, the excess

arsenite was titrated with standard (0.1N) iodine solution. The periodate uptake of lichenin was found to be as follows:

Time in hours.	Moles of periodate consumed/ $C_6H_{10}O_5$
24	0.692
48	0.712
72	0.725

The above results for the periodate consumption shows that the lichenin molecule lacks uniformity in the type of the linkages connecting the glucose units. Approximately 30% of the linkages are of the 1:3-type and the remaining 70% may be 1:4-, 1:2- or 1:6-type or a mixture of the three. The final answer to this question will no doubt be given by the trimethyl glucoses resulting from the hydrolytic fission of the fully methylated lichenin. This point will be discussed later on.

Hydrolysis of oxidised lichenin.

The completely oxidised lichenin was analysed for its glucose content. For this purpose, the oxidised lichenin (present in the last bottle) was filtered on a G3 sintered-glass crucible, washed with cold water until free from oxidant (tested with potassium iodide and sulphuric acid and diphenylamine and sulphuric acid) and then with alcohol, and dried in a

vacuum desiccator over phosphorus pentoxide. The dry oxidised lichenin (30 mg.) was hydrolysed with N-sulphuric acid (5 c.c.) in a sealed tube for 5 hours. The glucose content of the hydrolysed solution was then determined by the method of Flood, Hirst and Jones (J.C.S., 1679, 1948), using ribose as a reference sugar. The oxidised lichenin was found to contain 24.2% glucose, a result about 6% below the expected value. This discrepancy may be explained by the loss and distruction of the sugar involved in the several steps of the analysis.

Reducing-power of Lichenin.

The reducing power of lichenin (due to the reducing end group) will provide some information about the size of the lichenin molecule and two methods are now available for determining the reducing-power:

- (1) Hypiodite oxidation method
- and (2) Meyer's Colorimetric method.

Hypiodite oxidation method.

The estimation of aldoses in solution by quantitative oxidation to aldonic acid by alkaline solution of iodine has been referred to on many occasions by various authors. (Romihns, J.Soc.Chem.Ind., 16, 765, 1897; Bland and Lloyd, *ibid.*, 33, 948, 1914; Bougaut, Compt.rend., 51, 780, 1917; Willstatter and Schudel, Ber., 51, 780, 1918; Judd, J.Biochem., 14, 255, 1920; Kolthoff, Analyst, 48, 386, 1923; Hinter and Macara, *ibid.*, 49, 2, 1924; 52, 668, 1927; Kline and Acree, Ind.Eng.Chem.(Anal.), 2, 413, 1930, etc.). Ketoses are slightly attacked by the above reagent.

It has been suggested that the iodine first reacts with alkali to form iodide and hypiodite and that the latter is the effective oxidising agent. At the same time, varying degrees of alkalinity have been recommended by the different authors and varying conditions have been specified for the oxidation.

Ingles and Israel (J.C.S., 810, 1948) found a phosphate buffer of pH 11.3 at 25° to be an ideal one for the oxidation. Using carbonate-bicarbonate buffer (pH 10.6), Hirst, Hough and Jones (J.C.S., 928, 1949) got almost quantitative oxidation with both simple and methylated sugars. The obvious disadvantage of the carbonate-bicarbonate buffer is that there is a chance of losing iodine due to effervescence during acidification. Phosphate buffer does not suffer from this defect.

The reducing end-group of a polysaccharide may be estimated by hypoiodite oxidation but there are always some doubts about the quantitative aspect of this oxidation in the case of a polysaccharide. Possible sources of error are over-oxidation and side-reactions and hence the result from this method must always be accepted with some reserve.

The experimental procedure involved in the determination of the reducing power of lichenin by hypoiodite oxidation is described below.

A known weight of lichenin was treated with 10 c.c. of water in a cleaned stoppered flask and allowed to swell for 1-2 hours. 10 c.c. (accurately measured) of 0.1N iodine solution was added to the polysaccharide followed by an addition of 25 c.c. of the phosphate buffer (pH 11.4). The solution was then allowed to stand in the dark for 4-5 hours, acidified with 25 c.c.

of 2N sulphuric acid and the liberated iodine titrated with standard (0.1N) thiosulphite solution. Blank experiments were also carried out. The results are shown below:

Expt.1. Weight of lichenin = 74.14 mg.

Time of oxidation = 6 hours.

D.P. = 80 units.

Expt.2. Weight of lichenin = 125.2 mg.

Time of oxidation = 4 hours.

D.P. = 101 units.

Expt.3. Weight of lichenin = 70.93 mg.

Time of oxidation = 5 hours.

D.P. = 86 units.

The degree of polymerisation (D.P.) of lichenin was found to decrease with an increased time of oxidation which was possibly due to over-oxidation. It may be concluded from the above results that the D.P. is not less than 80 units.

Colorimetric method.

Meyer, Noelting and Bernfeld (Helv., 31, 103, 1948) have developed a method for the colorimetric estimation of reducing power (and hence the molecular weight) of a polysaccharide. The method depends on the interaction of the reducing group with 3:5-dinitrosalicylic acid in alkaline solution. It is suggested that the reaction involved is the quantitative reduction of 3:5-dinitrosalicylic acid (by the reducing

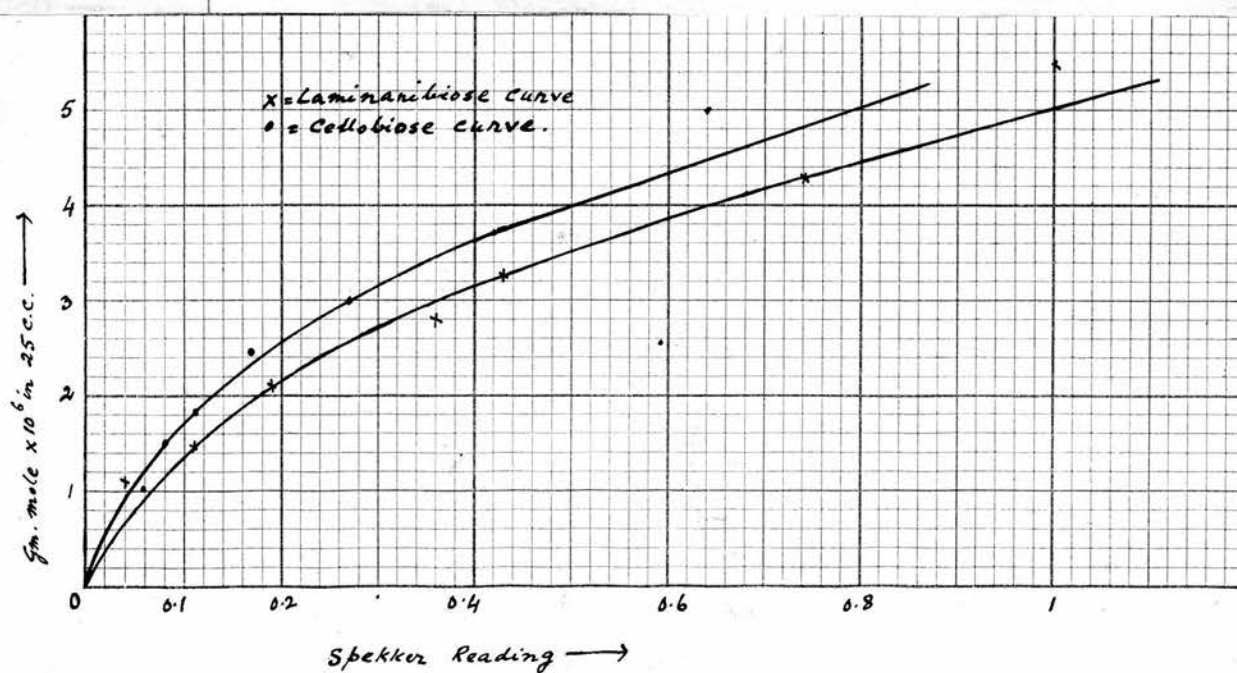
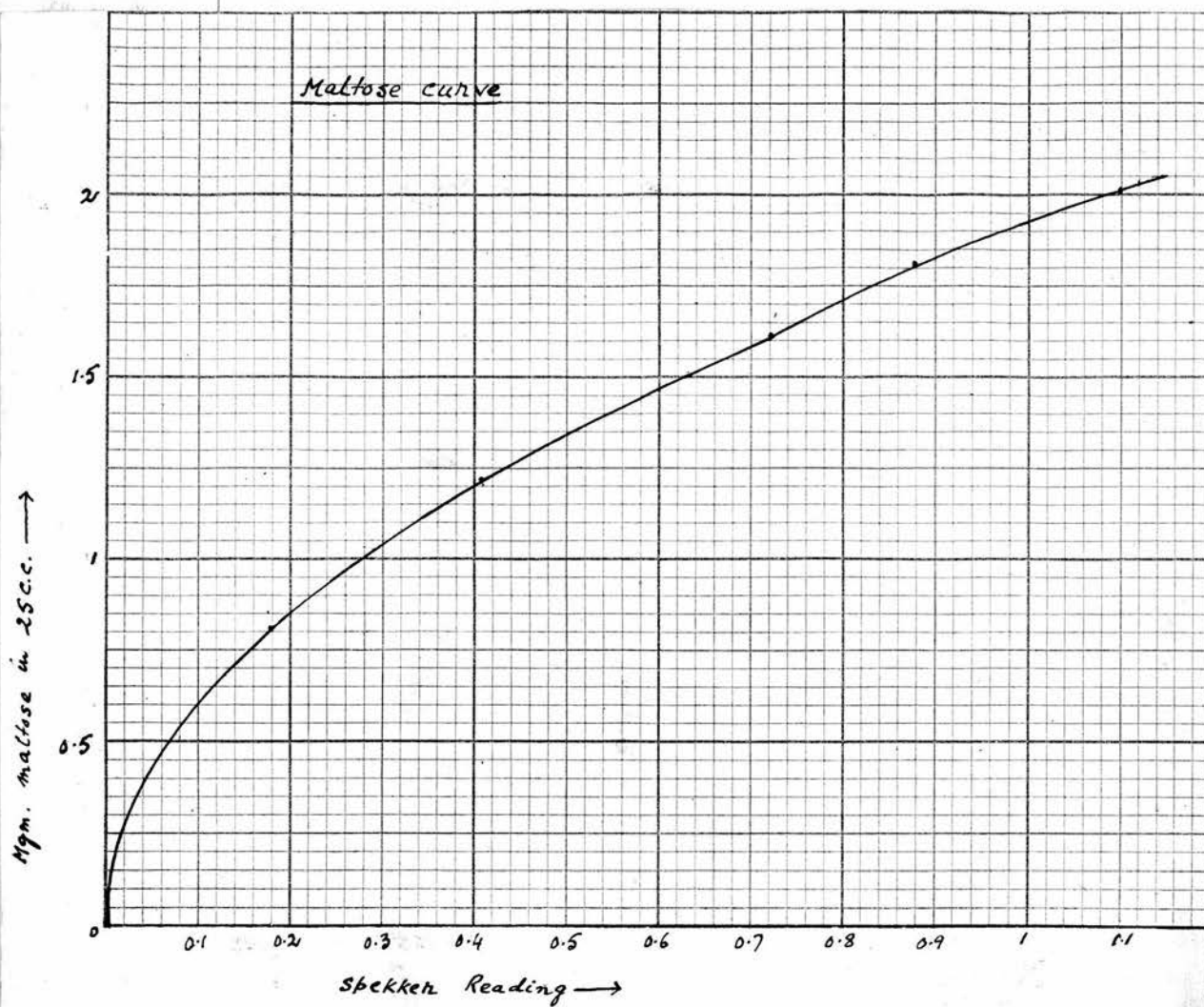
end-group) to amino-nitrosalicylic acid. Amino-nitrosalicylic acid, the reduction product, is coloured (orange) and its concentration is measured photometrically. A standard curve is prepared using maltose and the degree of polymerisation is given by relationship -

$$\text{D.P.} = \frac{2 \times \text{wt. of polysaccharide}}{\text{wt. of maltose}}$$

for two solutions having the same colorimetric absorption, i.e. the same reducing power. According to Meyer, the results from this method agree closely with results from other methods.

To determine the D.P. (degree of polymerisation) of lichenin, a standard curve of cellobiose was prepared by the following method.

10.c. of cellobiose solution (aqueous) containing a known amount of the sugar was treated with 1 c.c. of 1.5% 3:5-dinitrosalicylic acid and 1 c.c. of 6N caustic soda solution in a well-cleaned, dry test-tube. After adding 2 c.c. of water to the mixture to make the final volume 7 c.c., the solution was heated for 30 minutes at 65°. A blank experiment was also carried out simultaneously omitting the sugar. At the end of the reaction, the solution was cooled and diluted with water to 25 c.c. in a standard flask. The reduced solution was then compared against the blank in a "Spekker" photoelectric absorptiometer,



using 4 cm. cell and No.604 green filter. A series of observations with different concentrations (range: 1 to 2.5 mg. sugar) were made for the sugar. The results for the standard curves are tabulated below.

Cellobiose curve.

<u>Mg. cellobiose in 25 c.c. solution.</u>	<u>gm.mol. x 10⁶ in 25 c.c.</u>	<u>Spekker Reading (4 cm.cell)</u>
2.55	7.45	1.19
1.70	4.97	0.64
1.27	3.71	0.42
1.02	2.98	0.275
0.85	2.48	0.174
0.63	1.84	0.113
0.51	1.49	0.084
0.36	1.05	0.059

Maltose curve.

<u>Mg. maltose in 25 c.c. solution</u>	<u>gm.mole. x 10⁶ in 25 c.c.</u>	<u>Spekker Reading (4 cm. cell)</u>
2.0		1.10
2.02	5.90	1.10
1.81	5.29	0.88
1.61	4.70	0.728
1.21	3.53	0.418
0.81	2.67	0.183

* Laminaribiose curve.

<u>Mg. laminaribiose in 25 c.c. solution</u>	<u>gm.mol. x 10⁶ in 25 c.c.</u>	<u>Spekker Reading (4 cm. cell).</u>
1.86	5.43	1.01
1.49	4.35	0.74
1.12	3.27	0.434
0.96	2.80	0.359
0.74	2.16	0.194
0.50	1.46	0.117
0.38	1.11	0.047

* Quoted from the thesis of Dr. J.J. Connell.
(Edinburgh University)

The Spekker readings represented graphically against the concentrations of the different disaccharides gave the curves shown in the adjoining page.

Estimation of the D.P. of Lichenin.

Two samples (equal weight) of lichenin were first treated with 2 c.c. of water in two separate test-tubes and left for 2-3 hours to swell. Next, 1 c.c. of 6N caustic soda solution and 3 c.c. water were added to each of the test-tubes in order to get the polysaccharide in solution. To one of these solutions, 1 c.c. of 3:5-dinitrosalicylic acid (1.5%) was added (total volume 7 c.c.) and the other one, serving as a blank experiment, was treated with a further 1 c.c. of water. The dinitrosalicylic acid of the blank was measured out in a 25 c.c. standard flask. The two solutions were heated for 30 minutes at 65°C. (water-bath). After cooling at the end of the reaction, they were diluted to 25 c.c. in standard flasks. The two solutions were then compared in the Spekker absorptiometer as before (4 cm. cell; 604 green filter). Results were as follows:

Wt. of lichenin in mg.	Spekker reading	D.P. from cellobiose curve	D.P. from maltose curve	D.P. from laminaribiose curve
50.72	0.13	149	151	181
50.20	0.128	150	152	179

These results (considering the values from cellobiose

and maltose curves) show that lichenin has an apparent D.P. of 150 ± 2 and the value from the laminaribiose curve is relatively high. In fact, the above values are almost double the values obtained by the periodate and hypiodite oxidation methods and within the range of the values obtained by the viscosity measurements on the acetylated and methylated lichenin. With starch, laminarin etc., this method gives very low values for the D.P. which is probably due to degradation caused by sodium hydroxide (6N) during heating.

Acetylation.

In organic chemistry, the process of acetylation is of great importance in characterizing and estimating the free hydroxyl groups present in a compound. The sugars and their derivatives having free hydroxyl groups easily undergo acetylation either on treatment with acetic anhydride and pyridine at room temperature or heating with acetic anhydride and sodium acetate. The acetates having ester linkages are very sensitive to acids and alkalis and on account of this property they are of no use for structural studies. Nevertheless, sugar acetates are useful intermediates for synthetic work.

The acetylation is an easy and rapid reaction and the acetates are generally soluble in organic solvents. Study of some physical properties such as viscosity, osmotic and vapour pressures, etc. of polysaccharide acetates provides some information about the nature and size of the polysaccharide molecule. Acetolysis of polysaccharides, (the process of simultaneous hydrolysis and acetylation), carried out with a mixture of H_2SO_4 , acetic acid and acetic anhydride, gives rise to acetylated disaccharides (octaacetyl cellobiose from cellulose, octaacetyl maltose from starch, etc.), the characterisation of which provides a clue as to the nature of the repeating units in the polysaccharide molecule.

Lichenin acetate.

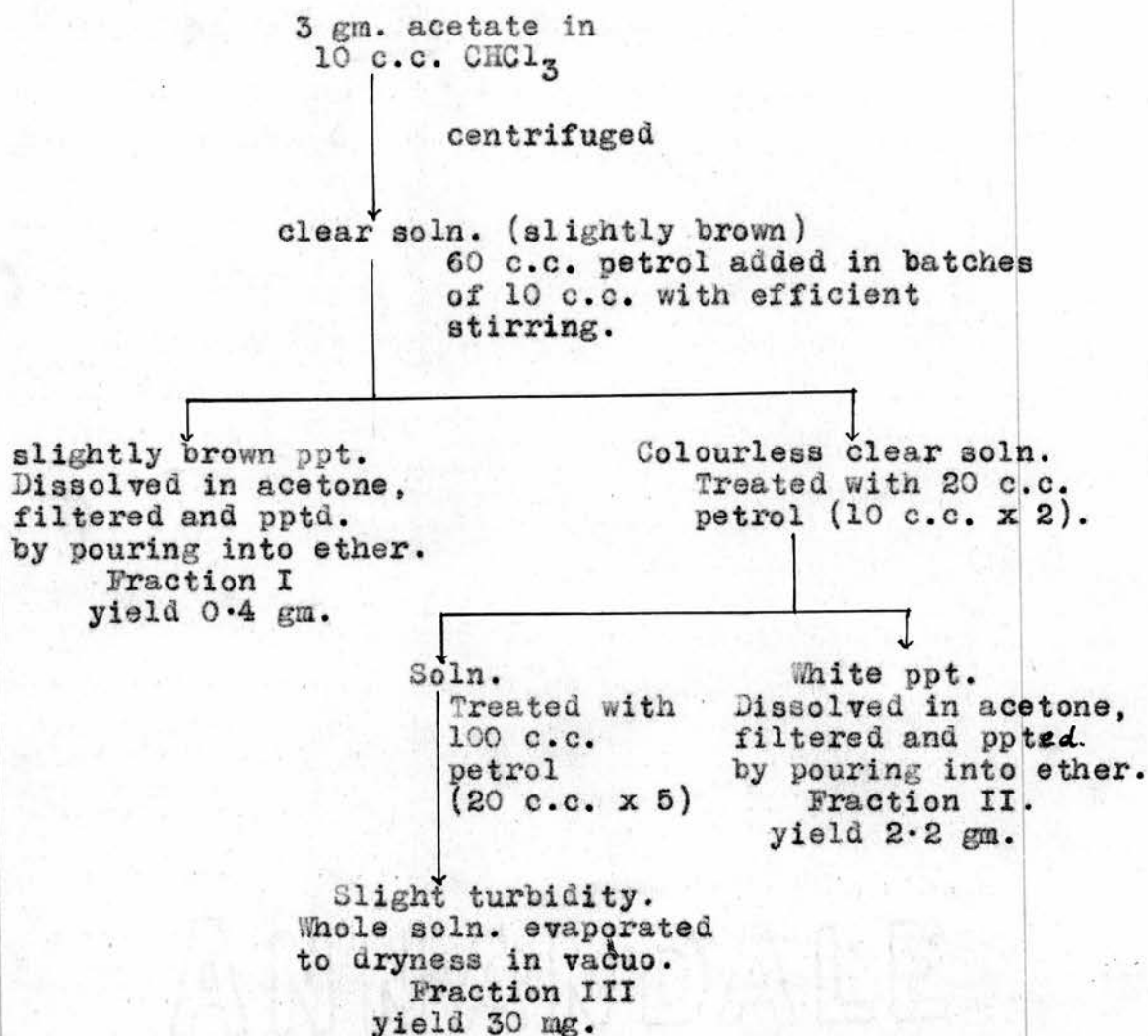
Lichenin was acetylated using acetic anhydride in the presence of pyridine by the method of Verley and Bölsing (Ber., 34, 3354, 1901). Esterification is not difficult if the lichenin is first carefully precipitated from solution and dried to a fluffy, non-horny powder, or if the lichenin is highly swollen and dispersed so as to make the polysaccharide molecules easily accessible to the esterifying reagents. For proper dispersion, lichenin (2.5 gm.) was first allowed to swell in 20 c.c. of water overnight and then treated with pure pyridine (30 c.c.) for 2-3 hours (Pacsu and Mullex, J.A.C.S., 1487, 1941). After this treatment, the mixture was subjected to vacuum distillation when an azeotropic mixture of pyridine and water distilled off. In this way, the last traces of water can be removed. When the first distillation was over, a few more c.c. of pyridine were added to the lichenin paste and the solution again subjected to vacuum distillation to ensure the complete removal of the last traces of water. In this way, lichenin was obtained in a thoroughly dispersed condition. The lichenin paste was then treated with pyridine (150 c.c.) and acetic anhydride (50 c.c.) added in small batches with shaking. The reaction began immediately, the lichenin going gradually into solution. The mixture was shaken for 30 hours at the

end of which time a clear solution was obtained.

Acetate was precipitated by pouring the mixture into ice-cold water and the acetate washed free from acid, pyridine with water and finally with methylated spirit, alcohol and ether in turn to remove water.

Lichenin acetate (3.4 gm.) was obtained in white fibrous state and was soluble in acetone, chloroform, pyridine, etc. The acetyl content of the product was found to be 44.03%. (Theory: 44.79%), Ash content: 0.5%.

The acetyl content of the acetylated product was determined by dissolving about 0.1 gm. in A.R. acetone (10 c.c.) and saponifying with approximately 0.1N sodium hydroxide solution (20 c.c.). The mixture and a blank were allowed to stand at room temperature for 10-12 hours with occasional shaking and then titrated with 0.1N hydrochloric acid. The fractionation of lichenin acetate was carried out by the precipitation method from a chloroform solution using petroleum ether (B.P. 40-60) as the precipitant. The procedure is indicated below:



The properties of lichenin acetate are summarised
in the table below:

Fraction	Physical state	Acetyl Content	Solvent	$[\alpha]_D^x$	
				Chloroform	Acetone
I	slightly brown, powdery.	43.8%	acetone chloro- form, pyridine.	-30.1° (c=1.1) x = 16°C	-13.62 (c=1.01) x = 18°C
II	white, fibrous	44.2%	acetone, chloro- form, pyridine.	-33.7° (c=1.03) x = 16	-12.4° (c=1.04) x = 16

Viscosity of lichenin acetate in m-cresol and chloroform.

The viscosity determination was carried out in an ordinary Ostwald viscometer and the degree of polymerisation (D.P.) was calculated from Staudinger's equation.

Fraction I in m-cresol.

Average time of flow	[Solvent = 705 sec.
at 20°C.		Solution = 1643 sec.
		(c = conc.gm.per litre = 9.42).

Therefore, $\eta_{sp}/c = 0.1412$

Hence, D.P. = 223, when $K_m = 6.3 \times 10^{-4}$ (Staudinger, et.al., Ann., 535, 95, 1938).

Fraction II in m-cresol.

Average time of flow	[Solvent = 476
at 20°C.		Solution = 1045 sec.
		(c = conc.gm.per litre = 10.97).

Therefore, $\eta_{sp}/c = 0.109$.

Hence, D.P. = 173, when $K_m = 6.3 \times 10^{-4}$

Fraction II in chloroform.

Average time of flow	[Solvent = 63 sec.
at 20°C.		Solution = 79 sec.
		(c = 10.12 gm.).

Therefore, $\eta_{sp}/c = 0.02509$.

Hence, D.P. = 47, when $K_m = 5.3 \times 10^{-4}$ (Staudinger and

Reinecke, Ann., 535, 95, 1938).

The different values of D.P. from the viscosity determinations in m-cresol and chloroform are difficult to explain and must be taken with reserve in view of the limited application of the Staudinger's equation.

Deacetylation of Lichenin acetate.

The periodate consumption of deacetylated lichenin was studied with a view to discovering whether the fractionation had proved effective in removing the 1,3-linked glucose residues from the main fraction (II), the former possibly existing in the original sample as an impurity.

The lichenin acetate (fraction II) was deacetylated by means of sodium-methoxide (Zemplen and Pacsu, Ber., 62, 1613, 1929). A solution of lichenin acetate (150 mg.) in chloroform (2 c.c.) was cooled in a freezing mixture and treated with 1 c.c. sodium-methoxide solution (100 mg. sodium in 5 c.c. absolute methanol). After shaking for 5 hours, the mixture was treated with ice-cold water (30 c.c.) to precipitate the deacetylated lichenin. The mixture was then neutralised with dilute acetic acid and the regenerated lichenin obtained in the dry state in the usual way.

Periodate uptake of deacetylated lichenin.

The periodate uptake of the deacetylated lichenin

was determined by the method previously described (page 32). The product consumed 0.68 mole of periodic acid per anhydroglucose residue which indicated the ineffectiveness of the fractionation in removing the 1,3-linked glucose residues. The four fractions of methylated lichenin (page 53) were also found to contain the two trimethyl glucoses, i.e. 2,4,6- and 2,3,6-trimethyl glucoses, in almost the same relative proportions. From all these observations, it was assumed that the 1,3-linked glucose residues form a part of the lichenin molecule.

Methylation.

In structural carbohydrate chemistry, methylation has proved to be of great importance - the process involves the replacement of the primary or secondary alcohol groups of a sugar by methoxyl groups. The true methyl ethers of the monosaccharides have been of the utmost service in two respects. In the determination of constitution, the stability of the ether link under drastic conditions of temperature, acidity, alkalinity, oxidation and reduction has provided an ideal means of 'masking' free hydroxyl groups, and the ease with which the methyl ethers of the sugars may be purified, by high vacuum distillation or chromatography technique followed by crystallization, gives them great importance as reference compounds and characterizing derivatives. On the other hand, the stability of the methyl ethers stands in the way of their use as intermediates in the chemical synthesis. In constitutional work, methylation of the unsubstituted alcoholic groups in a carbohydrate is followed by hydrolysis or oxidative disruption of the molecule and identification of the fragments produced. In this way, methyl ethers have proved to be of utmost importance in the determination of the ring structures of the monosaccharides and in the elucidation of the constitutions of the more complex

saccharides. In the study of polysaccharides, Haworth and Machemer (J.C.S., 2270, 1932) gave this method a quantitative aspect by developing the end-group method for the estimation of the chain-length.

Methylation is generally carried out by the use of dimethyl sulphate and sodium hydroxide solution (Haworth and Hirst, J.C.S., 8, 107, 1915), often followed by the use of Purdie's reagent (J.C.S., 1021, 1903), silver oxide and methyl iodide, for the completion of the reaction. Purdie's method is not applicable to a reducing sugar (unless the reducing group is protected) because of the oxidising action of the silver oxide, and even in Haworth's method it is necessary to adopt special precautions to prevent Lobry de Bruyn transformations (Rec.trav.Chim., 14, 203, 1895) and more extensive degradation due to the oxidation of the enolic forms. Other methods of more recent date, which have advantages in certain circumstances, include treatment of the carbohydrate in liquid ammonia with sodium and then with methyl iodide (Muskat, J. Amer.Chem.Soc., 56, 693, 2449; 1934; Hendricks and Rundle, *ibid*, 60, 2563, 1938; Freudenberg and Boppel, Ber., 41, 2504, 1938) and the use of thallous hydroxide and methyl iodide (Menzies, J.C.S., 937, 1926; Hirst and Jones, J.C.S., 502, 1938).

Methylation of lichenin.

Lichenin was methylated by the standard method of Haworth and Hirst, using caustic soda (30% solution) and dimethyl sulphate in an atmosphere of nitrogen to prevent oxidation of the polysaccharides. The reaction was carried out in a three-necked (3 l.) round bottom flask, fitted with a mechanical stirrer to stir the reaction mixture continuously during reaction.

On treatment with water (50 c.c.) the lichenin (6 gm.) initially swelled - this pre-swelling helps the solution of lichenin in caustic soda (30%). Dimethyl sulphate was added dropwise to the lichenin solution. A constant check was kept on the alkalinity of the reaction mixture, which was never allowed to become acid. After addition of 400 c.c. of dimethyl sulphate in 48 hours, the reaction mixture was neutralised with dilute acetic acid and dialysed to remove the inorganic salts. The partly methylated lichenin, obtained by concentrating the dialysed solution, was again subjected to the treatment of caustic soda and dimethyl sulphate (500 c.c. in 4 batches), with occasional addition of acetone to keep the partially methylated lichenin in solution. The partially methylated lichenin was regenerated by the method indicated above and subjected to the further action of caustic soda and dimethyl sulphate. In fact, seven



series of methylations were given to lichenin to get it in the highly methylated stage. It may be mentioned here that in the latter stage, the regeneration of the methylated polysaccharide was carried out by extraction of the neutralized solution (with dilute acetic acid) with chloroform. After seven methylations, a product was obtained, the methoxyl value of which was 40.78% and this value was not raised even on further treatment with caustic soda and dimethyl sulphate. Therefore, at this stage, a fractionation of the product was carried out.

During the purification of the methylated lichenin, one fact was observed regarding its precipitation: the methylated product can be precipitated with ligroin only from a benzene (dry) solution but not from acetone or chloroform solution.

Freshly precipitated methylated lichenin has the tendency to coalesce together and form a sticky mass. The purified methylated substance was obtained as a light brown solid soluble in benzene, chloroform, acetone and water.

Fractionation of Methylated Lichenin.

The methylated lichenin (OMe, 40.78%) was fractionated by the solution method, the solvent being a mixture of purified dry petroleum ether (75°-80° B.P.) and dry benzene. The amount of benzene in the extraction

mixture was increased stepwise.

Fraction	Solvent (300cc) Petrol:Benzene	Yield in gm.	-OCH ₃ %	$[\alpha]_D^{17}$ in CHCl ₃ .
1	95:5	0.076	41.5	-8.2
2	90:10	2.13	43.92	-8.1
3	85:15	1.34	43.12	-7.9
4	80:20	0.102	39.83	-

Relative viscosity of the fractions 2 and 3 in chloroform.

The relative viscosities of the fractions 2 and 3 in chloroform were measured and found to give practically the same values for η_{sp}/c .

$$\eta_{sp}/c \text{ (fraction 2)} = 0.01526$$

$$\eta_{sp}/c \text{ (fraction 3)} = 0.01688$$

Methylation of fractions 2 and 3 - Purdie's method.

The fractions 2 and 3 differed from one another very little in their values for optical rotation, methoxyl content and η_{sp}/c etc. Because of this small difference, they were combined and subjected to treatments with Purdie's reagent. After two treatments, the methylated lichenin was found to have a methoxyl value of 44.4% and an ash content of 0.81%. The rotation was found to be, $[\alpha]_D^{17}$ -8.21 (c, 0.74 in CHCl₃).

This product (OMe, 44.4%) was examined chromatographically: the development of the chromatogram showed

the presence of four spots corresponding to the four methylated glucoses: 2:3:4:6-tetramethyl glucose; 2:3:6-trimethyl glucose (brownish spot, R_G 0.83); 2:4:6-trimethyl glucose (pinkish spot, R_G 0.76) and dimethyl glucoses (more than one). The intensity of the spot corresponding to dimethyl sugar was very weak in spite of the fact that the original spotting was quite heavy and this faint spot of dimethyl glucose can easily be accounted for by demethylation during hydrolysis. From this observation, it was assumed that the polysaccharide was fully methylated though the observed methoxyl value was slightly lower than the theoretical. Two separate spots due to the two trimethyl glucoses, i.e. 2:3:6- and 2:4:6-trimethyl glucoses, were detected on running the chromatogram on a long paper (75 cm.) for 50 hours, this fact supported the result of periodate uptake regarding the presence of mixed linkages (1:3- and 1:4-types) in the lichenin molecule. The fractions 1 and 4 were also examined chromatographically and the examination revealed the presence of dimethyl sugars in appreciable amounts and this result was in disagreement with their methoxyl values.

Viscosity of methylated lichenin.

The viscosity of the methylated lichenin (OMe, 44.4%) in m-cresol and dry chloroform was determined in an ordinary Ostwald viscometer.

a) Average time of flow $\left[\begin{array}{l} \text{m-cresol (10 c.c.)} = 474 \text{ sec.} \\ \text{solution (10cc.c.)} = 1538 \text{ sec.} \\ (\text{c} = \text{conc. gm. per litre} = 11.29 \text{ gm}) \end{array} \right.$
 at 20°C.

Therefore, D.P. = 165, when $K_m = 12 \times 10^{-4}$

b) Average time of flow $\left[\begin{array}{l} \text{chloroform (10 c.c.)} = 62 \text{ sec.} \\ \text{solution (10 c.c.)} = 71 \text{ sec.} \\ (\text{c} = 8.32 \text{ gm.}). \end{array} \right.$
 at 20°C.

Therefore, D.P. = 14, when $K_m = 11 \times 10^{-4}$ (Staudinger, Ann., 535, 95, 1938).

Hydrolysis of the methylated lichenin.

Hydrolysis of the methylated lichenin to the free methylated sugar (glucose) involved two operations:

(1) First, the methylated lichenin was hydrolysed with methanolic hydrogen chloride to get the glycosides, and
 (2) the conversion of the glycosides into the corresponding reducing sugar by means of aqueous hydrochloric acid hydrolysis.

3 gm. of the fully methylated lichenin was treated with 1% methanolic-hydrogen chloride (150 c.c.) and refluxed gently on a water-bath. The hydrolysis was followed polarimetrically and at the end of 13 hours, the rotation became practically constant (+ 1.72). The acid solution was then neutralised with silver carbonate, filtered under gentle suction and the solid washed with hot dry methanol to remove the adhering glycosides. The methanolic solution of glycosides was carefully concentrated to a syrup.

The syrup contained some silver and no attempt was made to remove it at this stage.

The syrup (glycosides) was then subjected to hydrolysis by means of 0.5N hydrochloric acid (150 c.c.) and after 10 hours, the hydrolysis was found ($\alpha = +2^\circ$, constant) to be complete. The acid solution was cooled to room temperature and neutralised with silver carbonate. The solution was filtered and treated with hydrogen sulphide gas to precipitate the silver still present in solution. The solution was again filtered through a well-washed charcoal/filter-cell bed under suction and a clear filtrate obtained. The filtrate was then concentrated in vacuum to a syrup. The syrup was found to be associated with some silver salts and these were removed by extracting the syrup with chloroform. The chloroform solution was finally concentrated in vacuum to a clear brownish syrup (3.2 gm.).

The syrup was a mixture of tetra-, tri, and di-methyl glucoses, possibly contaminated with some glycosides. The next step in the study of this mixture involved its separation into the different methylated glucoses and this was achieved on a cellulose column (Hough, Jones and Wadman, *Nature*, 162, 448) by means of the partition chromatography technique.

Estimation of the methylated glucoses.

The relative proportions of the different methylated sugars present in the hydrolysed syrup was determined by the method of quantitative paper chromatography. For this purpose, the different sugars were separated on a paper and estimation was carried out in the standard way. It may be mentioned here that quite heavy spotting was necessary to determine the position of the end-group and also to obtain a measurable amount. This heavy spotting did not interfere with the separation.

The amount of different sugars *were*:

<u>Per cent composition</u>		
2,3,4,6-tetramethyl glucose:	0.122 mg.	1.11
Trimethyl glucose:	10.49 mg.	97.85
Dimethyl glucose:	0.11 mg.	1.02

The chain length of lichenin calculated on the above result indicates one non-reducing end group per 86 units.

Chromatography.

In the study of structural polysaccharide chemistry, the earlier workers have used the method of 'Fractional distillation' (under reduced pressure), to separate the glycosides obtained on the methanolysis of the methylated polysaccharide and the various fractions obtained are examined for refractive indices, methoxyl contents, rotations and characterized by the preparation of various derivatives. Fractional distillation, however, has disadvantages in that pyrolysis, demethylation, incomplete separation and non-quantitative recovery of the sugars are unavoidable. In addition, relatively large quantities of material are required. Recently, all these defects have been eliminated by the development of "Chromatography" technique, which may be considered as an ultra-filtration method capable of separating the molecules.

Chromatography has proved of the utmost value in the separation of colouring matter of plants, of vitamins, of hormones and of various other complex organic compounds such as fatty acids, dyes and nucleic acids etc. Attempts have also been made (by Reich, Colman, Wolfrom, Mertzweiller, etc.) to extend its application to mixtures of sugars by preparing coloured compounds such as the phenyl azo-benzoyl esters. Such a method depends on the production

of coloured bands on a column of alumina, silica gel or other adsorbent at different points of the column after developing with a suitable solvent. This method suffers from the defect that the preparation and subsequent decomposition, after separation, of the coloured esters involve the chances of losing the material.

Bell (J.C.S., 473, 1944) and Jones (J.C.S., 333, 1944) succeeded, using a partition method between chloroform and water held in a silica gel column, in separating tetramethyl glucose (50 mg.) from 10 gm. of trimethyl glucose. Methylated sugars fluoresce in ultra-violet light (Norberg, Auerbach and Hixon, J.A.C.S., 67, 342, 1945), this property was also used in following the movement of sugars in the column. The fully methylated sugars are absorbed least strongly, since no free hydroxyl groups are present and this band passes down the column first.

Martin, Synge, Consden and Cordon developed the technique of "Partition Chromatography" for the separation of amino-acids from the hydrolysis of proteins, using filter paper as the supporting medium. Some idea of the efficiency of the method may be obtained from the separation and identification of 22-amino acids from only 400 μ g. of hydrolysed wool protein. In all biological research, this technique has proved of great success. It has been found

possible to extend the technique to sugars. Partridge (Nature, 158, 270, 1946; J.Biochem., 42, 238, 1948) used filter-paper strips, the top ends of which dip into a trough containing n-butanol saturated with water in an atmosphere of butanol and water vapour, the apparatus being maintained at a uniform temperature. He showed that mixtures of several sugars could be separated on a micro-scale and identified by the rate of travel along the paper chromatogram. With different spraying agents, the sugars are identified on the paper. Flood, Hirst and Jones (Nature, 160, 86, 1947; J.C.S., 1679, 1948) gave this method a quantitative aspect for separating and estimating different simple and methylated sugars on a micro-scale. Such mixtures of sugars can also be separated on a large scale by the use of a column of powdered cellulose, (Hirst, Jones and Wadman, Nature, 161, 720, 1948; Hough, Jones and Wadman, *ibid*, 162, 448).

Separation of the methylated glucoses.

A column of powdered cellulose (90 cm. x 2.5 cm.) was prepared, washed and tested as described by Hough, Jones and Wadman (*loc.cit.*). The solvent employed in the separation was a water saturated mixture of 60% purified petroleum ether (B.P. 100°-120°) and 40% purified n-butanol. The column was first washed with this solvent before putting in the sugar mixture.

The syrup obtained on hydrolysis of the methylated lichenin was dissolved in 3-4 c.c. of the solvent and the solution added dropwise to the centre of the top of the column, each drip being allowed to soak in before the next drop was added. After transferring all the syrup into the column, it was left for 2-3 hours to reach an equilibrium condition. The top reservoir was then filled with solvent and the elution process began. The eluate was collected in tubes. An automatic device changed the tubes at a suitable time interval.

The distribution of the sugars in the tubes was determined by paper chromatography in the usual way. In all, 375 tubes were collected, each containing 4-5 c.c. solvent. The distribution of the methylated sugars in the tubes was found to be as follows:

Tubes	1 - 27	contained	tetramethyl glucose.
"	34 - 144	"	trimethyl glucose.
"	177 - 327	"	dimethyl glucose.

The contents of the tubes were then combined according to the distribution of sugars, the solvent removed under vacuum, and the residue dissolved in water and filtered through a charcoal/filter-cell bed to remove colour and waxy impurities. The aqueous solution was evaporated to dryness under reduced pressure, the residue dissolved in freshly distilled acetone (A.R.) and filtered to remove the residual

impurities. The sugar was obtained by removing the acetone under reduced pressure. At the end of the separation experiment, the column was washed with water and the aqueous extract evaporated to dryness and the residue purified in the usual way. Paper chromatography examination of the residue revealed the presence of traces of free glucose and monomethyl glucose, which probably originated from "demethylation."

The yield of the different sugars were as follows:

Tetramethyl glucose fraction :	0.4554 gm.
Trimethyl glucose fraction : (mixture of 2:3:6- and 2:4:6-trimethyl glucoses)	2.4690 gm.
Dimethyl glucose fraction :	0.039 gm.
"Water-washed" fraction :	0.015 gm.

Total 2.9784 gm.

Purity of the different fractions.

The purity of each fraction was determined by the hypiodite oxidation method and the results obtained are tabulated below:

Tetramethyl glucose fraction :	10.3%
Trimethyl glucose fraction :	91.5%
Dimethyl glucose fraction :	85.1%

The amount of each sugar obtained, calculated on the basis of the above per cent. of purity are as follows:

1. Tetramethyl glucose : 0.047 gm.
2. Trimethyl glucose : 2.6438 gm.
3. Dimethyl glucose : 0.033 gm.

Total 2.7238 gm.

Per cent. of total recovery 90.7%

Examination of the different fractions.

1. Tetramethyl glucose fraction. This fraction was obtained in a syrupy condition which failed to crystallise even on long standing - this syrupy condition together with the high weight of the fraction (0.4554 gm.) indicated that the 2:3:4:6-tetramethyl glucose was contaminated with some trimethyl-methylglycosides. When a small test sample of the syrup was rehydrolysed and examined chromatographically, the presence of trimethyl glucose along with the tetramethyl glucose was indicated.

The amount of the 2,3,4,6-tetramethyl glucose in this fraction was determined by the alkaline hypiodite oxidation method and 47 mg. (10.3%) was found to be present in the mixture. The chain length of lichenin calculated on this result, i.e. 0.047 gm. of tetramethyl glucose from 3 gm. methylated lichenin, indicates one non-reducing end-group per 73 anhydro glucose units.

Rehydrolysis and separation of the tetramethyl glucose fraction.

The fraction (0.4554 gm.) containing tetramethyl

glucose and trimethyl-methylglycosides was rehydrolysed with N-hydrochloric acid (12 c.c.) and the partly crystallised sugar mixture was separated in the usual way on a cellulose column. In this case, a small column (70 cm. x 2 cm.) was used and after separation, the different sugars were recovered as described previously. The amount of the tetramethyl and trimethyl glucoses obtained were as follows:

2:3:4:6-tetramethyl glucose : 32 mg.

Trimethyl glucose : 410 mg. (88.5% pure)
(mixture of 2 trimethyl
glucoses)

The tetramethyl glucose was obtained in a crystallised condition on keeping for a few days in a refrigerator. The trimethyl glucose fraction was also obtained in crystalline state. The tetramethyl glucose was purified by crystallisation from pure dry light petroleum ether.

2. Trimethyl glucose fraction.

The periodate uptake of lichenin indicated the presence of mixed linkages (1:3- and 1:4-linkages) in the lichenin molecule - this observation was verified by the presence of two different trimethyl glucoses, 2,3,6- and 2,4,6-trimethyl glucoses, in the trimethyl glucose fraction resulting from the hydrolysis of the methylated lichenin. The R_G values of the two trimethyl glucoses (2:3:6-trimethyl glucose = 0.83; 2:4:6-trimethyl glucose = 0.77) are very close and a

separation of the two sugars in a long paper (75 cm.) was achieved by running it for 50 hours. The two trimethyl glucoses give spots of quite different colours with aniline oxalate: 2:4:6-trimethyl glucose gives a reddish pink spot while the other sugar, 2:3:6-trimethyl glucose, appears as a brown spot.

Hydrolysis of a portion of this fraction and subsequent investigation on a chromatogram did not reveal the presence of any other sugar except traces of dimethyl glucose probably due to demethylation. This fraction was obtained in a crystalline condition and the hypiodite oxidation indicated its purity to be 91.5%.

Search for 2,3,4-trimethyl and 3,4,6-trimethyl glucoses in the trimethyl glucose fraction.

Lichenin was found to consume periodic acid up to 70% and this uptake may be due to the presence of 1:4, 1:6 or 1:2 linkage in the molecule. The final answer to these possibilities will be provided by the different trimethyl glucoses coming from the fully methylated lichenin. The trimethyl fraction was critically examined for 2:3:4-trimethyl and 3:4:6-trimethyl glucoses. The 2:3:4-trimethyl glucose (R_G 0.85) is a syrup and its absence in the mixture was proved by running the trimethyl glucose fraction in a chromatogram against an authentic specimen of 2:3:4-trimethyl glucose which appears as a reddish-pink

spot with aniline oxalate and which separates in a well-defined way from the other trimethyl glucoses. The trimethyl glucose mixture was also examined by the Weerman reaction following the standard procedure but no precipitate of hydrazodicarbonamide was obtained. This negative Weerman reaction indicated the absence of the 3:4:6-trimethyl glucose in the mixture. From these results, it follows that the linkages in the lichenin molecule are composed of 1:4- and 1:3-types only.

3. Dimethyl fraction.

The dimethyl fraction (39.2 mg.) was found by the hypiodite oxidation method to be 85.13% pure, i.e. the fraction contains 33.3 mg. of dimethyl sugars.

Qualitative chromatographic examination indicated that the fraction consists of 2:3-dimethyl glucose and some other dimethyl glucoses, possibly 2:6-, 3:6-dimethyl glucoses, etc. The different dimethyl glucoses are difficult to separate at all, except the 2:3-dimethyl glucose which separates in an ill-defined way from the rest if a long paper (75 cm.) is used for a 50 hours run. This fact together with the small amount of the fraction made a thorough analysis of the mixture by means of Bell's method (J.C.S., 992, 1948) difficult.

An attempt was made to find approximately the relative proportions of 2:3-dimethyl glucose and the

other dimethyl glucoses (2:6-, 3:6-dimethyl glucoses, etc.) present in the mixture by the method of quantitative paper chromatography (Hirst, Jones, Flood, J.C.S., 1949, 1948). For this purpose, the impure sample was first purified by treatment with charcoal and subsequent solvent extraction and then analysed by the method mentioned above. The analysis indicated the presence of 53% 2:3-dimethyl glucose (17.6 mg.) in the mixture together with 47% of the other dimethyl glucoses (15.7 mg.).

Separation of 2:3:6-trimethyl and 2:4:6-trimethyl
glucoses.

2:3:6-trimethyl glucose (R_G 0.83) and 2:4:6-trimethyl glucose (R_G 0.76) run very closely, a fact that is evident from their R_G values and their separation is quite a difficult process. An attempt was made on a sample of 500 mg. of the mixture, using a long column (90 cm. x 2.5 cm.) and a water saturated petroleum ether n-butanol (1:1) mixture as a solvent. The distribution of the two sugars after the first separation was as follows:

Up to 106 tubes = pure 2:3:6-trimethyl glucose.

107 - 121 tubes = mixture.

122 - 142 tubes = pure 2:4:6-trimethyl glucose.

The sugar present in the tubes 107-121 was recovered and passed through the column again. This time also two pure fractions of the two trimethyl glucoses and an intermediate mixed fraction were obtained. By passing the mixed fraction through the column twice the following amounts of the two pure trimethyl sugars were finally obtained.

Pure 2:3:6-trimethyl glucose : 333 mg.

Pure 2:4:6-trimethyl glucose : 129 mg.

and mixture : 18 mg.

from 500 mg. of the mixture.

Periodic acid oxidation of 2:3:6-trimethyl glucose.

2:3:6-trimethyl glucose contains a pair of adjacent cis-hydroxyl groups and so one would expect it to be oxidised quantitatively by periodic acid but with 2:4:6-trimethyl glucose, the reverse is true. Thus it should be possible to estimate quantitatively the 2:3:6-trimethyl glucose in a mixture of 2:3:6- and 2:4:6-trimethyl glucoses by oxidation with periodic acid.

In an attempt to apply the above principle to the analysis of the mixture of the two trimethyl glucoses (2:3:6- and 2:4:6-) obtained from the methylated lichenin, it was found that only 10-12% of the sugar is attacked at room temperature and this value does not change even on allowing the oxidation to continue for 4-5 days. The same type of result (14-15%) was also obtained with a pure authentic specimen of the 2:3:6-trimethyl glucose. This abnormal behaviour of 2:3:6-trimethyl glucose towards periodic acid caused us to study the oxidation in detail, the temperature of oxidation and the concentration of the periodate being varied. Percival and Lund (unpublished) found the following results in a preliminary work on this problem.

Temp. C°.	Periodate concn.	Time in hrs.	% oxidation
15	0.25M	96	14.4
30	0.25M	388	85.8
40	0.3M	77	87.6
50	0.3M	28]	81.8]
		48]	131.0]
60	0.3M	4]	31.6]
		24]	139.0]

The above results show that the oxidation does not proceed beyond 14% at room temperature. An increase of temperature however has a marked effect which no doubt involves over-oxidation. The obvious reasons for this abnormal behaviour of the 2:3:6-trimethyl glucose is associated with the stability of the pyranose ring in the acid medium. In the case of methylated xylose, Percival and Chanda (unpublished) found 100% oxidation of 2:3-dimethyl xylose. It is of interest to note here that ambiguous behaviour of the partially methylated aldoses towards periodate was also observed by Hirst and Jones (J.C.S., 1659, 1949) and also by Bell (J.C.S., 992, 1948). This problem will be studied again using a buffered alkaline medium following the suggestions of Reeves (J.A.C.S., 63, 1476, 1941).

Rotation of the trimethyl glucose fraction in 2% methanolic-hydrogen chloride.

In order to obtain an approximate idea of the

relative amounts of the two trimethyl glucoses (2:3:6- and 2:4:6-) in the trimethyl fraction, its rotation in cold 2% methanolic hydrogen chloride solution was observed and compared with that of an artificial mixture of known composition of the two sugars in the same solvent.

The rotational change was as follows:

$$[\alpha]_D = +36.3^\circ \longrightarrow -15^\circ, \text{ const. in 24 hours (c = 1.04)}$$

The rotational change with an authentic mixture of 68.4% 2:3:6-trimethyl glucose and 31.7% 2:4:6-trimethyl glucose was found as follows:

$$[\alpha]_D = +57.2^\circ \longrightarrow -12.6^\circ, \text{ const. in 24 hours (c = 1.08)}.$$

The original equilibrium composition of the trimethyl fraction from the column was disturbed by the removal of about 0.4 gm. of sugar as glycoside to the tetramethyl glucose fraction. This fact possibly explains the discrepancy (2.5°) between the above two equilibrium rotations.

Characterisation of the different methylated glucoses.

1. 2:3:4:6-Tetramethyl-d-glucose.

The 2:3:4:6-tetramethyl-d-glucose was purified by crystallisation from pure dry light petroleum (B.P. 40°-60°) and had m.p. 83°-86°, $[\alpha]_D^{15}$, +83° at equilibrium (c, 0.67 in water), C, 51.2%, H, 8.5%, OMe, 50.8%, ($C_{10}H_{20}O_6$ requires C, 50.9%, H, 8.5%, OMe, 52.5%)

2. 2:3:6-Trimethyl-d-glucose.

Pure 2:3:6-trimethyl glucose was obtained by crystallisation from pure dry ether and had m.p. 120°-122°C., $[\alpha]_D^{16}$, +67.4° at equilibrium (c, 0.53 in water), C, 48.4%, H, 8.39%, and OMe, 40.9% ($C_9H_{18}O_6$ requires C, 49.1%, H, 8.1%, OMe, 41.9%).

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Phenylhydrazide of 2:3:6-trimethyl-d-glucose.

The 2:3:6-trimethyl glucose was characterized by making its phenylhydrazide $C_5H_8O_2(OCH_3)_3.CO = NH.NH.C_6H_5$ which has a sharp melting point (145°C.). It was prepared by the method of Haworth, Hirst and Carrington, (J.A.C.S., 55, 1084, 1933).

The trimethyl sugar was first oxidised by means of bromine at 35°C. for 24 hours. At the end of oxidation, the excess bromine was removed by aeration and the solution extracted with chloroform. Removal of chloroform under vacuum, left a yellow syrup which on distillation gave 2,3,6-trimethyl- -gluconolactone

as a syrup. The phenylhydrazide was prepared by heating a mixture of the lactone and freshly distilled phenylhydrazine (slight excess of molar proportion) on water-bath for 20 minutes. At the end of the reaction, the excess phenylhydrazine was removed by washing with benzene. Pure phenylhydrazide was obtained by recrystallisation (3 times) from ethyl acetate.

The pure phenylhydrazide had m.p. $145^{\circ}\text{C}.$, OMe, 27.67, C, 54.38%, H, 7.22%, N, 8.25%. ($\text{C}_{15}\text{H}_{24}\text{O}_6\text{N}_2$ requires C, 54.9%, H, 7.3%, N, 8.6%, OMe, 28.4%).

3. 2:4:6-Trimethyl-d-glucose.

Crystallisation from pure dry ether gave pure sample of 2:4:6-trimethylglucose. The pure sample had m.p., $119^{\circ}\text{-}123^{\circ}\text{C}.$, $[\alpha]_{\text{D}}^{17}$, $+74.3^{\circ}$ at equilibrium (C, 0.82 in water), C, 48.37%, H, 8.34%, OMe, 40.5% ($\text{C}_9\text{H}_{18}\text{O}_6$ requires C, 49.1%, H, 8.1%, OMe, 41.9%).

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2:4:6-Trimethyl glucose anilide.

The sugar (25 mg.) and freshly distilled aniline (20 mg.) were refluxed in absolute alcohol (10 c.c.) for 2 hours and the solvent removed under reduced pressure. The excess of aniline was removed by washing the residue $2/3$ times with light petrol containing 10% alcohol and this petrol mixture (hot) also used for recrystallisation. On recrystallisation (3 times) of the residue, white needles m.p. $143\text{-}144^{\circ}$

were obtained.

It may be noted here that the m.p. of the anilide recorded in literature is 162-6°C. (Granichstäden, Percival, J.C.S., 54, 1943). The small amount of the anilide did not permit further analysis.

Enzymatic hydrolysis of Lichenin.

Lichenin is easily attacked and converted into sugars by enzymes, the so-called "Lichenases," which are widely spread in plants and invertebrate animals. Some attempts have been made to study the action of lichenase on lichenin but different workers reported different results. Pringsheim and Leibowitz (Z. Physiol.Chem., 131, 262, 1923) claimed a 100% conversion of lichenin into cellobiose using a lichenase preparation obtained from malted barley and they cited the observed rotatory power ($[\alpha]_D^{20} = +31.4$) of the hydrolysed lichenin solution as a proof of the presence of 100% cellobiose (in the solution). Karrer and his co-workers (Helv., 7, 518, 1924) reported 100% conversion of lichenin into glucose using a freshly prepared snail juice extract (from *Helix pomatia*) as the enzymatic agent. After ageing for a month, the same preparation of enzymes was found (Karrer, et.al., *ibid.*, 154) to liberate a different sugar (possessing a very low reducing power) from lichenin which the above authors could not identify. In the present approach to this problem, the action of two lichenase preparations was studied giving different results from those cited above.

Preparation of enzyme solution.

The enzyme (lichenases) solutions from two different

sources:

(1) malted barley
and (2) snail juice (*Helix aspersa*),
were prepared and purified, as far as possible, by
the procedure indicated below.

(1) Lichenase from malted barley. This was prepared
by the method of Euler and Svanberg (*Z. Physiol. Chem.*,
112, 193, 1921). 100 ~~gms~~ of powdered malted barley
were extracted for 24 hours with 400 c.c. water by
shaking in a stoppered flask. The extracted solution
was then filtered to give a clear liquor which was
dialysed for 2-3 days against distilled water. The
dialysed solution, free from all free sugars was
stored for 5 months to destroy the other enzymes
present, especially the cellobiase (Pringsheim, et.al.,
loc.cit.).

(2) Lichenase from *Helix aspersa*.

The intestinal juice of *Helix pomatia* or *Helix aspersa* contains "lichenases" along with several other enzymes such as invertase, lipase, diastase, inulase, maltase, cytase, cellobiase, etc. Dialysis of the juice for 2-3 days against distilled water removes all the enzymes except the lichenase and some cellobiase. The enzyme solution is freed from cellobiase also on ageing for a month. (Karrer, et.al., loc.cit.). According to this method, a lichenase preparation was made from the intestinal juice of *Helix aspersa*.

Action of lichenase solution (malted barley) on lichenin.

Before proceeding with an actual experiment with lichenin, the enzyme solution was tested for its cellobiase content using a solution of cellobiose, The presence of ^avery small amount of cellobiase was deduced from the appearance of a very weak spot of glucose on the paper chromatogram. In the case of lichenin, the experimental details are given below:

2 c.c. 1% lichenin solution *] Incubating temp. 37°-39°C. Time, 48 hours.
0.5 c.c. acetate buffer (pH=4.6)	
0.5 c.c. enzyme solution	
1 drop toluene	

* Lichenin solution was made by warming (50°C).

At the end of the incubation period, salts are removed from the solution by treatment with Amberlite resins. On examination on a paper chromatogram (developed with aniline oxalate) run in pyridine-water-butanol-benzene solvent, spots were identified representing glucose, laminaribiose and an unidentified trisaccharide, together with two other spots which were no doubt due to oligosaccharides. A weak spot corresponding to cellobiose was also found on the paper. It may be noted here that laminaribiose travels faster than cellobiose (in pyridine solvent) and separates with a gap of about 4 cm. in 48 hours. The intensity of the different spots suggested that the proportions of

glucose and laminaribiose are almost the same and they, together, constitute about 60% of the sugar content of the solution and the cellobiose together with the trisaccharide and oligosaccharides account for the rest (40%). A similar experiment with an incubation period of 4 hours gave weak spots corresponding to glucose, laminaribiose and some of the higher saccharides but no cellobiose.

Action of lichenase solution (malted barley) on laminarin.

Soluble laminarin was also subjected to the action of the lichenase solution under exactly the same conditions as used in the case of lichenin. In this case spots corresponding to glucose, laminaribiose and some oligosaccharides were detected on the chromatogram. A weak spot corresponding to cellobiose was also recorded on the chromatogram, the origin of which was no doubt due to the presence of some 1,4-linked glucan as an impurity in the laminarin sample used.

Action of snail juice on lichenin.

Even on storing for 2 months, the snail juice was found to contain a considerable amount of cellobiase. With lichenin under the conditions described previously, this enzyme solution gave only glucose in appreciable amount. On ^achromatogram, no other spot except that of glucose could be detected.

These results of the action of lichenase on lichenin will be discussed later on.

DISCUSSION.

The lichenin used in the present investigation was obtained from Iceland moss (*Cetraria islandica*) where it occurs as a cell-wall component. The properties of this sample of lichenin agree with those ascribed by Schulze to a hemicellulose. It is a white powder, soluble in dilute alkalis, giving a clear colloidal solution with boiling water and easily hydrolysed to glucose with dilute acids at ordinary pressure. If heated in a vacuum, it forms laevoglucosan (Karrer and Nichida, *Helv.*, 7, 363, 1924; *ibid.*, 129).

Lichenin was extracted from the moss by means of boiling water and the crude product gave a blue colour with iodine indicating the presence of some isolichenin, another polymer of glucose found in Iceland moss. Polysaccharides, giving rise to mannose, galactose and a pentose sugar on hydrolysis, were also found to be associated with the lichenin. The purification of lichenin was carried out by repeated fractional precipitation from hot water solution and the pure product was obtained in the form of a white powder with a negative colour reaction with iodine indicating the absence of isolichenin. No uronic acid could be detected in the pure sample either by paper chromatogram or by the naphthoresorcinol test.

Hydrolysis of pure lichenin with N¹ sulphuric acid in a sealed tube was complete in 2-3 hours when 94%

glucose was found polarimetrically and 95% reducing hexose by hypiodite oxidation. The neutralized hydrolysate when examined by the method of paper chromatography showed the presence only of glucose, thus verifying that lichenin is composed wholly of D-glucose residues.

Lichenin possesses a positive specific rotation, $[\alpha]_D$, less than 10° , in aqueous sodium hydroxide (N), whereas the methylated and acetylated materials have negative rotations. This fact together with a comparison of the rotations of other glucans and their derivatives with lichenin and its derivatives points to the presence of β -glucosidic links in the lichenin molecule.

Cellulose Laminarin Lichenin Starch Glycogen

Glucosidic Link	β	β	β	α	α
Methyl derivatives	-10.0 - 4.7	-4.4	-8.0	+208	+208
Acetyl derivative	-22	-52	-30 to -33	+163	+163
Polysaccharide	-3.5	-7 to -16	8 to 10	+190	+192

The idea of the β -configuration has also received support from the result obtained by a study of the kinetics of the hydrolysis of lichenin with 51%

sulphuric acid. Drake (Biochem.Z., 313, 328, 1942) found the hydrolysis velocity of lichenin to be half of that of starch and double that of cellulose. Compared with cellobiose, maltose undergoes cleavage (Freudenberg, Dürr, and Hochstetter, Ber., 61, 1735, 1928) about 1.5 times faster. All those facts favour the above view of the β -configuration.

To elucidate the constitution of lichenin, the method of methylation was employed. Seven methylations at room temperature (in a nitrogen atmosphere) by the method of Haworth gave a product of about 40% methoxyl content and this value was not raised on further treatment with the methylating reagents. At this stage, the methylated lichenin was fractionated by the "solution method." Out of the four fractions (page) obtained, fractions 2 and 3 were found to be similar as indicated below:

Fraction	-OCH ₃ %	$[\alpha]_D^{17}$ in CHCl ₃	η_{sp}/c
2	43.92	-8.1	0.01526
3	43.12	-7.9	0.01688

In view of the small differences in the various properties of the two fractions, they were combined and subjected to two treatments with Purdie's reagent when a product of 44.4% methoxyl content was obtained. The product had, $[\alpha]_D^{17}$, -8.21 in CHCl₃(c, 0.74), and an ash content of 0.81%. A chromatographic examination of the product showed the presence of four spots

corresponding to the four methylated glucoses:
2:3:4:6-tetramethyl glucose; 2:3:6-trimethyl glucose;
2:4:6-trimethyl glucose and dimethyl glucose (or more
than one). The intensity of the spot corresponding
to dimethyl sugar was very weak despite the fact that
the original spotting was made quite heavily. The
appearance of this amount of dimethyl sugar in the
hydrolysed syrup may be due to demethylation and under
methylation to a certain extent. The remaining
fractions (1 and 4) similarly examined showed appreci-
able amounts of dimethyl glucose, a fact in agreement
with their methoxyl contents.

The product (3 gm.) of methoxyl content 44.4%
was then hydrolysed in the usual way to a mixture of
methylated glucoses. The amount of the different
sugars in the syrup was estimated by hypiodite oxi-
dation after separation on a paper chromatogram and
was found to be in the following proportions:

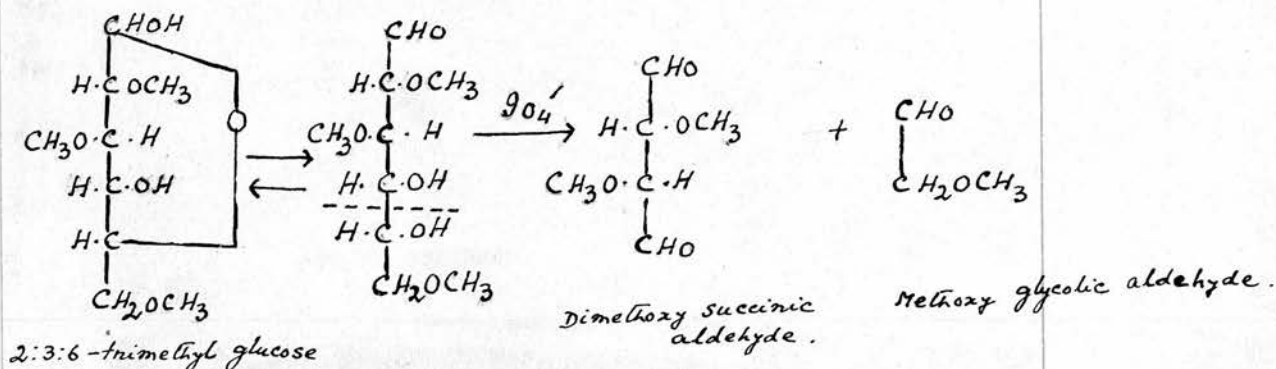
Tetramethyl glucose:	1.11%
Trimethyl glucose:	97.85%
Dimethyl glucose:	1.02%

The oxidation was carried out in caustic soda-
phosphate buffer (pH = 11.4). The chain length cal-
culated from the above composition indicated one non-
reducing end-group per 86 glucose units.

A lower value (D.P. 73) was obtained when the
methylated glucoses were separated on a cellulose

column on a macro-scale. By this method, the tetramethyl glucose fraction (0.4554 gm.) was obtained in the form of syrup which was proved to be due to the presence of some trimethyl-methylglycoside present along with the tetramethylglucose. The main trimethyl fraction (2.469 gm.) obtained in a crystalline state, was found to contain two trimethyl glucoses, i.e. 2:4:6-trimethyl glucose and 2:3:6-trimethyl glucose, a fact in agreement with the periodate uptake result. Hypiodite oxidation of the end-group fraction indicated the presence of 47 mg. of pure tetramethyl glucose which corresponds to a chain length of about 73 units.

The trimethyl glucose fraction was a mixture of 2:3:6-trimethyl and 2:4:6-trimethyl glucoses. To determine the composition of the mixture, the periodate oxidation method was resorted to, but the method did not prove successful. The 2:3:6-trimethyl glucose, having a pair of adjacent cis-hydroxyl groups, is



expected to undergo quantitative oxidation by periodic acid but in practice, only 10-12% oxidation was

obtained at room temperature. At higher temperature, over-oxidation was observed. This abnormal behaviour of the 2:3:6-trimethyl glucose is probably connected with the stability of the pyranose ring in the acid solution. An approximate idea of the amount of 2:3:6-trimethyl glucose in the mixture was obtained from a comparison of the final equilibrium rotation of the mixture in 2% methanolic-hydrogen chloride with that of an artificial mixture of 68.4% 2:3:6-trimethyl glucose and 31.7% 2:4:6-trimethyl glucose in the same solvent. The artificial mixture showed a specific rotation of -12.5° ($c = 1.08$) whereas the unknown mixture gave a value of -15° ($c = 1.06$).

The agreement was considered to be reasonable in view of the fact that the original equilibrium composition of the trimethyl fraction was disturbed by the removal of 0.408 gm. of the trimethyl sugar from the main fraction (during first separation) as glycosides in the end-group fraction. By repeated separation of the mixed trimethyl fraction on a cellulose column (p. 68), 333 mg. of 2:3:6- and 129 mg. of 2:4:6-trimethyl glucoses were obtained in a pure state from 500 mg. of the mixture. It may be noted here that this separation by repeated passage through the column involves considerable loss of sugar. Taking this loss into account, this result together with the observed equilibrium specific rotation (-15°) in 2%

methanolic-hydrogen chloride indicated the approximate composition of the mixture to be 70% 2:3:6-trimethyl glucose and 30% 2:4:6-trimethyl glucose. The 2:3:4-trimethyl glucose (R_G 0.85) is a syrup and separates perfectly from the other trimethyl glucoses on a chromatogram and the absence of this sugar in the trimethyl glucose mixture was proved by a negative chromatographic test. The mixture (first oxidised with bromine to acid and then converted into the amide) was also examined for the 3:4:6-trimethyl glucose by the Weerman reaction but no precipitate of hydrazodicarbonamide was obtained. These negative tests eliminated the possibility of the existence of 1:6- and 1:2-linkages in the lichenin molecule.

In structural polysaccharide (hexosan) chemistry, the role of dimethyl sugar is important from the point of view of branching in the molecule. The branching point will always give rise to a dimethyl sugar although its presence does not always denote branching in view of the fact that demethylation and under-methylation sometimes account for the appearance of dimethyl sugar and, in fact, these two phenomena, i.e. demethylation and under-methylation, cannot be avoided altogether in practice. Working with a pure specimen of 2:4:6-trimethyl glucose, Connell, Hirst and Percival, (J.C.S., 3494, 1950) found 4.7% demethylation for a 7 hours hydrolysis with 2N-hydrochloric acid and 8.7%

demethylation for 11 hours hydrolysis with the same acid. Bell (J.C.S., 992, 1948) has noted 2% demethylation of 2:3:6-trimethyl glucose when the pure methyl glycoside was hydrolysed for 5 hours with a mixture of glacial acetic acid and approximately N-hydrochloric acid. MacWilliam and Percival (J.C.S., 2259, 1951) reported 1% demethylation of 2:3:6-trimethyl glucose by the action of 1% hydrochloric acid. In the present investigation, qualitative experiments (N-HCl; 3 hours) on demethylation of pure samples of 2:4:6- and 2:3:6-trimethyl glucoses showed appreciable amounts of dimethyl glucoses with traces of monomethyl glucose. From the methylated lichenin about 1% dimethyl glucose (33 mg.) was obtained and shown to be a mixture of several dimethyl glucoses. An approximate analysis of the fraction showed that the mixture contained about 53% (17.6 mg.) of 2:3-dimethyl glucose. Other dimethyl glucoses, possibly 2:6-, 3:6-dimethyl glucoses, etc., which do not separate on a paper, constitute the remainder of the mixture (15.7 mg.). Theoretically in a singly branched chain with one non-reducing end, the amount of the dimethyl sugar should be in equivalent proportion to the amount of the end-group whereas in the case of a chain with two non-reducing ends, the equivalent proportion of the dimethyl sugar should be half of that of the tetramethyl glucose. According to this theoretical consideration,

42 mg. of dimethyl glucose (considering 47 mg. end group) should originate from a branched chain of two reducing ends and 21 mg. should come from a branched chain of two non-reducing ends. The amount experimentally obtained (33 mg.) is in between the above two theoretical values and so this result does not give any definite information. While considering the phenomenon of demethylation, the amount of dimethyl glucoses (1%) is within the observed demethylation range of 2:3:6- and 2:4:6-trimethyl glucoses. From this consideration, the appearance of about 1% dimethyl glucoses can be accounted for by demethylation and some under-methylation. Though the phenomenon of demethylation can account for the dimethyl sugar, the possibility of a branched molecule is not ruled out altogether until the accurate molecular weight of lichenin is forthcoming from the osmotic and vapour pressure methods. From the specific viscosity of methylated and acetylated lichenin, an approximate idea of the molecular size may be obtained, though, in the absence of any definite knowledge of the molecular shape, it is impossible to make valid application of the Staudinger equation:

Degree of polymerisation (D.P.)

$$= \eta_{sp}/c.Km$$

where η_{sp} = specific viscosity of the solution, e.g. the viscosity increase over that of the solvent.

c = concn. in gm. per litre.

K_m = a constant.

The equation is based upon a number of suppositions.

Unfortunately, they are seldom strictly fulfilled.

The three basic assumptions made by Staudinger in deriving this equation are noted below: (1) the solution of the polymer contains the material in the form of single molecules:

(2) the viscosity measurements are independent of the nature of the solvent and that the phenomenon of solvation does not substantially interfere with the viscosity.

(3) the chain molecules represent stretched-out, rod-shaped units and that these "thread molecules" retain their shape in solution.

All these assumptions are open to question and have been strongly criticized by various authors (Kuhn, McBain, Liepatoff, et al.).

The specific viscosity of methylated lichenin in *m*-cresol showed the D.P. to be 165 ($K_m = 14 \times 10^{-4}$). A set of divergent values was obtained for the lichenin acetate.

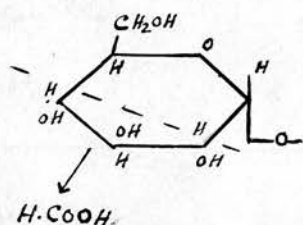
Lichenin acetate I	- 223	} <i>m</i> -cresol, when $K_m = 6.3 \times 10^{-4}$
Lichenin acetate II	- 173	
Lichenin acetate II	- 47	(in CHCl_3), when $K_m = 5.3 \times 10^{-4}$

These values for D.P. are much higher than the values

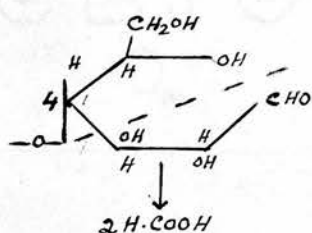
obtained by the different chemical methods. In view of the various limitations of the viscosity equation, the above values must be regarded with some doubt.

The structural information obtained from the examination of the different methylated glucoses has been substantiated by the result given by the periodic acid oxidation of lichenin. Lichenin was found to consume about 0.7 mole of periodic acid per anhydro-glucose unit which means that about 30% of the linkages in the lichenin molecule are 1,3-type and the remaining portion (70%) may be 1,4-, 1,6-, or 1,2- or a mixture of the three. The final answer to this question has been obtained from a study of the trimethyl glucose fraction which contained only 2:3:6- and 2:4:6-trimethyl glucoses proving thereby that only 1,4 and 1,3-linkages are present in the lichenin molecule. This fact of mixed linkages differentiates lichenin from cellulose where the linkage is uniformly 1,4-type.

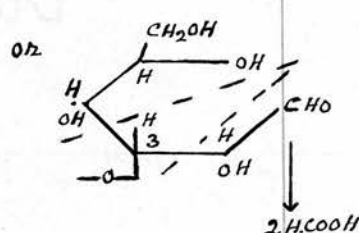
During periodic acid oxidation, two moles of formic acid will be liberated from the reducing end of



Non-reducing end



Reducing end



lichenin and one mole from the non-reducing end. Lichenin was found to give 0.0333 moles of formic acid per $C_6H_{10}O_5$ unit. Assuming lichenin to be a straight chain polymer, the above amount of formic acid corresponds to a chain length of 90 units, a value in close agreement with the one obtained from the end-group assay. In the case of a uniform 1,4-linkage polysaccharide, information may be obtained about branching in the molecule from an examination of the residue left after periodic acid oxidation. Periodic acid will not attack the branching unit devoid of α -glycol grouping and this residue appears as a free sugar when the oxidised material is hydrolysed. The method has proved helpful in the case of starch, glycogen, xylan, etc., but is of no use in the present case because of the presence of mixed linkages (1:4 and 1:3) in the molecule.

Hypoiodite oxidation of lichenin has given a value of about 80 units for the D.P. This method sometimes suffers from the drawback of over-oxidation and due to this fact, the above result suggests that the D.P. is not less than 80 units. Estimation of the reducing group by colorimetric measurement of the reduction of 3:5-dinitrosalicylic acid to nitro-amino-salicylic acid by the method of Meyer, et.al., gave a value of the order of one reducing group per 150 units, when reference is made to the standard

cellobiose and maltose curves. With respect to the laminaribiose curve, the degree of polymerisation turns out to be 180. The order of these values is the same as those found from viscosity determinations of methylated lichenin and lichenin acetate II in m-cresol. In view of the several limitations of this colorimetric method, these results must be taken with some reserve.

The actual size and nature of the lichenin molecule cannot be represented here in the absence of an accurate value for the molecular weight of the polysaccharide. For the present if the presence of a single chain molecule, is assumed, the results from the end-group assay, periodate and hypoiodite oxidations, suggest the chain length of lichenin to be of the order of 80 ± 10 .

In the case of a branched molecule, the chain may be terminated by two non-reducing and one reducing group or two reducing and one non-reducing group. If the lichenin molecule is assumed to be singly branched with one reducing and two non-reducing groups, the end-group assay shows the chain length to be 146 whilst the periodate oxidation result suggests a chain length of 120 units. The end-group assay value (146) is in close agreement with those obtained from the viscosity and colorimetric methods.

All the above discussion is based upon the

assumption that the lichenin preparation is homogeneous in character - homogeneous in the sense that the 1,3-linked glucose residues (30%) form a part of the molecule and are not present as an impurity in the form of a separate 1,3-glucosan chain. Fractionation of the methylated and acetylated lichenin in an attempt to remove the 1,3-linked residues, the latter possibly existing as an impurity, did not produce any positive result. Lichenin acetate (fraction II), after deacetylation, was found to consume only 0.68 mole of periodic acid per $C_6H_{10}O_5$. Similarly, all the fractions of the methylated lichenin on hydrolysis showed two spots on the chromatograms corresponding to the 2:3:6- and 2:4:6-trimethyl glucoses and the intensities of the two spots indicated almost similar relative proportions of the two trimethyls in the four fractions. These facts show that either the fractionation was ineffective or that both types of linkages (1,4 and 1,3) are present in the molecule. A true answer to this question is rather difficult. If lichenin were an α -glycosidic polysaccharide, a study of the activity of β -amylase might have given us some information in this direction.

The action of the so-called 'lichenase' enzyme on lichenin has been studied with a view to obtaining some information in this direction from the nature of the cleavage products. Various previous investigators

have reported different results from the enzymatic study of lichenin. Pringsheim and Leibowitz (Z. Physiol.Chem., 131, 262, 1923) reported a 100% conversion of lichenin into cellobiose by the action of a lichenase preparation from malted barley whereas Karrer, et.al., observed a 100% conversion of lichenin into glucose working with freshly extracted juice of snails (*Helix pomatia*). On ageing for a month, the same preparation of enzymes was found (Karrer, et.al.) *Helv.*, 7, 154, 1924) to give a sugar of a very low reducing power which the authors could not identify. The results obtained in the present investigation are different from both those cited above. The hydrolysed lichenin solution was found to contain glucose and laminaribiose in almost equal proportion (glucose and laminaribiose, 60% of the total) together with some oligosaccharides (tri-, tetra-, penta-, etc.) and a very small amount of cellobiose (p. 77). An old preparation of the enzymes from snail juice produced only glucose in appreciable amount from lichenin.

These results are hard to evaluate in terms of the action of a single enzyme in view of the heterogeneous character of the enzyme preparation. Lichenase has never been isolated in the pure state and its mode of action on the lichenin molecule is completely unknown. Further, the action of lichenase is likely to be influenced by the associated constituents. In

this connection, the conversion of cellulose into glucose by means of cellulase is worth considering here although the route of break-down is not fully known yet.

Pringsheim reports (Z.Physiol.Chem., 137, 265, 1924; *ibid.*, 78, 266, 1912) that the hydrolysis of cellulose by cellulase proceeds through the intermediate formation of cellobiose, and that a cellobiase is also present which continues the hydrolysis to glucose. It is claimed that the cellulase is more stable to higher temperatures than the β -glucosidase and by conducting the hydrolysis at 67°C., an accumulation of cellobiose takes place. Grassmann, et.al. (Grassmann, Zechmeister, Toth and Stadler, Ann., 503, 167, 1933; *ibid.*, 502, 20, 1933) found the action of cellobiase is not confined to the cleavage of cellobiose but that it is capable of splitting also the oligosaccharides, i.e. cellotriose, cellotetraose and cellohexaose. However, its activity ceases with cellodextrin. On the other hand, cellulase seems chiefly to hydrolyse fragments with a higher degree of polymerisation. Cellulase thus splits the cellulose chain no further than to about the hexaose stage, after which further cleavage is brought about by the cellobiase.

A similar type of action may be exerted by lichenase on the lichenin molecule in view of the fact

that the hydrolysed lichenin solution was found to contain appreciable amounts of oligosaccharides besides glucose, laminaribiose and the small amount of cellobiose. The laminaribiose no doubt originated from the 1:3-linked glucose residues of the lichenin molecule. Recently Dillon and O'Colla (*Nature*, 166, 67; *Chemistry and Industry*, 111, 1951) have reported the presence of laminarinase in extracts of wheat, oats, barley, potato and hyacinth bulbs. These extracts also hydrolysed lichenin to glucose and yeast glucan partially to glucose and partially to laminaribiose. The enzyme solution used in this investigation also contains some of the so-called 'laminarinase' since, while working with laminarin, it gave glucose, laminaribiose and some oligosaccharides.

The route of break-down is not clear from these results. It is more likely that the lichenin molecule, under the influence of the various enzymatic factors present in the preparation, undergoes a random hydrolysis to various oligosaccharides, followed by further cleavages to the mono- and di-saccharides. The appearance of appreciable amounts of laminaribiose is no doubt due to break-down of the 1:3-linked glucose residues (30%) - these residues may exist as a separate chain (impurity) in the preparation or as a part of the lichenin molecule forming one of the two ends. In both cases, the laminarinase will attack them producing laminaribiose. There is another

possibility and that is that the 1:3-linked residues are distributed in pairs among the 1:4-linked residues in the chain in such a way that they appear as laminaribiose as a result of the random cleavage produced by lichenase.

A true answer to these possibilities will go a long way to the understanding of the lichenin molecule and for this purpose, attention should be directed to the study of the different oligosaccharides.

Summary.

1. Lichenin was extracted from Iceland moss (*Cetraria islandica*) and an impure preparation gave a greenish-blue colour with iodine indicating the presence of some isolichenin, another polysaccharide found in the same moss. Chromatographic examination of pure lichenin showed that the polysaccharide is composed of glucose units only.
2. The small positive rotation of lichenin (below 10°) together with the negative rotations of the methylated and acetylated materials indicated the presence of β -glucosidic links in the lichenin molecule.
3. Lichenin consumed about 0.7 mole of periodic acid per anhydroglucose residue which means that about 30% of the linkages in the lichenin molecule are 1:3-type and the remaining portion (70%) is 1:4-type.
4. During periodate oxidation, lichenin gave 0.0333 mole of formic acid per anhydroglucose unit, a result which corresponded to a chain length of about 90 units.
5. The reducing power of lichenin (hypoiodite oxidation) corresponded to a molecular size of 80 units, whilst reaction with 3:5-dinitrosalicylic acid gave a much higher value (150) with reference to maltose and cellobiose curves.
6. Methanolysis and hydrolysis of methylated lichenin yielded a mixture of sugars consisting of 2:3:4:6-

tetramethyl glucose, 2:3:6-trimethyl glucose, 2:4:6-trimethyl glucose, 2:3-dimethyl glucose and some other dimethyl glucoses, possibly 2:6-, 3:6-dimethylglucoses; etc., which do not separate on a chromatogram. The presence of the two trimethyl glucoses (2:3:6- and 2:4:6-trimethyl glucoses) was in agreement with the periodate uptake result.

7. The end-group assay indicated the chain length of lichenin to be about 73 units.

8. About 1% dimethyl glucose was obtained from the methylated lichenin - this amount of dimethyl sugar was within the observed demethylation range of 2:3:6- and 2:4:6-trimethyl glucoses.

9. The chain length of lichenin, calculated from viscosity measurements (in m-cresol) of the methylated and acetylated materials, was found to be in the range 160-80.

10. The actual size and nature of the lichenin molecule cannot be decided without an accurate value of the molecular weight of the polysaccharide. For the present, if a linear molecule is assumed the results from all the chemical methods indicate the chain length of lichenin to be 80 ± 10 .

11. Lichenase, an enzyme preparation from malted barley, produced glucose and laminaribiose in almost equal proportion together with some oligosaccharides and a very small amount of cellobiose. A preparation

of lichenase from snail juice (*Helix aspersa*) produced only glucose from lichenin. These results are difficult to explain in view of the present lack of knowledge of the properties of 'lichenase' and the heterogeneous character of the enzyme preparation.

PART II. ISOLICHENIN.

ANNANDALE

POLTON

Introduction.

Isolichenin, which accompanies lichenin in very small amounts, is very soluble in water (even in ice-cold water) and has the characteristic property of acquiring a blue colour with iodine, a property indicating a structural relationship with amylose. According to König and Schuberth (Mont., 8, 452, 1887), isolichenin differs from lichenin in being destroyed by the action of malt extract at 60°C. - it also differs in being stable (Karrer and Staub, Helv., 7, 159-62, 1924) towards lichenase, an enzyme specific for the hydrolysis of lichenin. A most striking property of isolichenin is its high dextro-rotatory power in aqueous solution, whereas lichenin has a positive specific rotation below 10° in N-sodium hydroxide solution.

Since the later part of the last century, several attempts have been made to study the nature of isolichenin with practically no success. In 1887, König and Schuberth (loc.cit.), while studying lichenin, became interested in isolichenin and obtained four fractions by the method of fractional precipitation whose specific rotations (in water) lie in the range + 102.8° to 145.6° and also recorded some colour reactions of these fractions with iodine. König was not sure of the absence of lichenin from all his

fractions. From this study, the authors claimed that isolichenin is similar to a soluble modification of ordinary starch. In 1920, Salkowski (Z. Physiol. Chem., 110, 158, 1920) claimed a separation of lichenin from isolichenin and also found the latter to give a blue colour with iodine like amylose. These authors did not attempt to investigate the problem of the actual constitution of isolichenin and their conclusions are entirely based on the high rotation of isolichenin and its characteristic blue colour with iodine solution.

The next attempts to solve this problem were made by Pringsheim and Karrer and in fact, for some time they fought each other to defend their respective points of view on the problem of the constitution of isolichenin. Pringsheim (Ber., 57, 1581, 1924) claimed the isolation of isolichenin as a white powder (with small ash content) from Iceland moss by hot-water extraction and subsequent repeated freezing and thawing of the concentrated liquor to remove the last traces of the accompanying lichenin, and finally the isolichenin was precipitated with alcohol. He observed its rotation to be the same as amylose and managed to effect a quantitative splitting to maltose by means of freshly prepared malt extract. The slow action of emulsion on isolichenin as in the case of amylose was also observed by Pringsheim. This fact of quantitative

conversion of isolichenin to maltose by malt-extract indicates the absence of lichenin because in that case cellobiase (present in malt-extract) would have converted lichenin into glucose. Maltose was identified as ^acrystalline nitrate. From this observation, Pringsheim postulated that isolichenin has the same structure as amylose. This observation of Pringsheim was immediately contradicted by Karrer. Karrer and Joos (Z.Physiol.Chem., 141, 311-315, 1924) took up this question and obtained quite different results from those of Pringsheim by attacking the problem in a different way. They isolated isolichenin from Iceland moss and then subjected it to fractionation by means of Fehling's solution, whereby they obtained three fractions possessing the following properties:

Fraction A. Non-reducing to Fehling's solution, gives no colour with iodine, is very soluble in hot water (less in cold water) and has a positive rotation, (specific rotation = + 88°). A moderately concentrated aqueous solution gives a blue precipitate with Fehling's solution, the formation of which is greatly helped by heating. It has the following composition:

Mannose - about 21%, estimated by means of phenylhydrazine.

Galactose - about 35%, estimated as mucic acid.
and about 45% of another carbohydrate, consisting mainly of glucose.

Fraction B. Slightly reducing to Fehling's solution, gives very weak blue colour with iodine, is highly soluble in cold water, gives no precipitate with Fehling's solution and is more dextro-rotatory than the fraction A (specific rotation = + 148°) and contains only glucose.

Fraction C. The amount was so small that it did not permit any investigation.

From this work, Karrer and Joos concluded that isolichenin was a mixture of various polysaccharides and at the same time challenged the observation of Pringsheim regarding the quantitative conversion of isolichenin to maltose.

Following on this work, Pringsheim (Z. Physiol. Chem., 144, 241-245, 1925) prepared more of the pure isolichenin of specific rotation + 188.2° (in water) and forwarded a sample to Karrer for verification of his observation. Karrer found the same rotation and a precipitate with Fehling's solution but, according to him, this agreement of rotation and reducing power with that of maltose was purely accidental and there was no justification for assuming the presence of 100% maltose from these facts. It may be mentioned here that Karrer did not hydrolyse the sample of Pringsheim and he also ignored the fact about the identification of maltose by Pringsheim as the crystalline nitrate. With Fehling's solution, isolichenin gave a precipitate

(according to Karrer) and Pringsheim explained it by referring to the same phenomenon with amylose and thereby he reiterated his original claim.

Karrer (Z.Physiol.Chem., 148, 62-64, 1925) again took up this question and isolated some isolichenin from Iceland moss according to the method of Pringsheim. Isolichenin was then hydrolysed with 5% hydrochloric acid and the neutralized sugar solution was treated with phenylhydrazine hydrochloride and sodium acetate. Within 10 minutes time, crystals of mannose-phenylhydrazone (yield - 5% of the weight of isolichenin) was obtained. At this stage, Karrer repeated the same experiment on the sample sent by Pringsheim and obtained mannose phenylhydrazone (m.p. 196°), yield - about 6% of the weight of isolichenin. Armed with these facts, Karrer became sure of the heterogeneity of isolichenin and again criticized the observation of Pringsheim. At this point the controversy was dropped and, in fact, nobody took up the question until quite recently (1947), Meyer and G"urtler (Helv., 30(1), 761, 1947) made an attempt to throw some light on the nature of isolichenin. Meyer, et.al., followed the general principle of the method of Pringsheim in extracting and purifying isolichenin. By means of an elaborate fractionation process using Fehling's solution, they finally obtained four fractions possessing quite different properties. The properties of these fractions are summarised below:

Fraction	Sugars	Non-fermentable sugars	$[\alpha]_D^{15}$	$[\alpha]_D^{15}$ after hydrolysis	Solubility in water	Blue- colour with J_2 soln.	Reaction with Fehling's soln.	Splitting with β -amylase
A	glucose 100%	nil	+9.6° in 2N-NaOH	+41° in H_2O	0	+	0	0
B	glucose <100%	13%	+35° in 2N-NaOH	-	+	+	+	0
C	glucose, mannose and galactose	nil	+109° in H_2O	-	+	0	+	0
D	glucose <100%	6%	+203° in H_2O	+38.5° in H_2O	+	0	0	0

According to Meyer, isolichenin is a mixture of at least four different polysaccharides, a result similar to that of Karrer.

The above review of the literature of isolichenin reveals that the question of the constitution of isolichenin is still unsolved in spite of several attempts in this direction. The complex character of the polysaccharide is quite evident from the presence of three different sugars. In the present investigation, an attempt has been made to discover the exact nature of this complex polysaccharide with the help of the chromatographic technique, which has proved very successful in sugar chemistry.

EXPERIMENTAL.

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Extraction and purification of isolichenin.

A review of the literature revealed that all the previous investigators on isolichenin have been confronted with difficulties in obtaining the polysaccharide in a pure state and this was the main reason for the varied results leading to the great controversy about the real nature of the polysaccharide. Karrer, Meyer, et.al., showed it to be a heterogeneous polysaccharide while Pringsheim claimed it to be a homogeneous substance. So the first essential in the study of isolichenin is the isolation of the substance in a pure state. In the present investigation, a method has been developed for obtaining the polysaccharide in a pure state - pure in the sense that it did not give any other sugar, on hydrolysis, except glucose. The possibility of the present preparation being a mixture of different glucose polysaccharides is not excluded altogether - this is a point difficult to prove with the methods of purification available at present.

The brown mother liquor left after the removal of the separated lichenin (p. 24) was used for the preparation of isolichenin, whose presence was indicated by the development of a blue colour with iodine solution. This liquor still contained some lichenin (about 10% of the total) and its removal involved the first step in the purification of isolichenin.

Isolichenin is soluble even in ice-cold water, this property has been of service in its purification.

The brown liquor was first concentrated to a small volume in vacuum and centrifuged to remove the separated polysaccharide. The clear concentrated liquor was then frozen to a solid mass with the help of an ice-salt mixture. The frozen mass was allowed to melt slowly at room temperature when lichenin came out of the solution partially. This process of freezing and subsequent melting was repeated 3-4 times to ensure the complete removal of lichenin and finally the liquor concentrated under diminished pressure to a small volume and then centrifuged to remove the separated polysaccharide from the brown liquor. Isolichenin was precipitated from this brown solution with acetone in the form of a brown sticky mass which on drying assumed a brown horny structure. This product was then dissolved in cold dilute hydrochloric acid (1%), centrifuged to remove the insoluble residue (lichenin) and the acid solution treated with acetone to precipitate the isolichenin. The precipitated isolichenin was made acid free by washing with 50% acetone - and then with acetone, alcohol and ether in turn to remove the water and dried in a vacuum desiccator over phosphorus pentoxide. This product was brown in colour and had $[\alpha]_D^{17} = 165^\circ$ in water ($c = 0.62$). This was named isolichenin A. A chromatographic examination

of this preparation revealed the presence of glucose in major amount together with some galactose, mannose and a pentose sugar, possibly arabinose. A faint spot corresponding to a uronic acid was also detected on the chromatogram. The next step in the purification therefore involved the removal of the polysaccharides giving rise to the associated sugars (galactose, mannose, etc.).

It was observed that the isolichenin-copper complex, when shaken with water, partly dissolved leaving behind one-third of the whole as a residue. The soluble and insoluble fractions were separated and the polysaccharides regenerated from the two fractions. A qualitative chromatographic examination of the hydrolysates of the two fractions revealed the distribution of sugars in following order:

Insoluble fraction: mainly mannose and galactose
with some glucose.

Soluble fraction: mainly glucose with small
amounts of mannose, galactose and the pentose sugar.

The specific rotation of the soluble fraction was found to be about 212° in aqueous solution ($c = 0.4$).

The solubility of the isolichenin-copper complex in water was utilized in the second step of the purification process. The copper complex of the crude preparation was shaken with water for 2-3 hours

and the soluble and insoluble fractions separated by centrifuging. After filtration through a No.3 sintered glass funnel, the copper-complex was acidified with dilute acetic acid (to decompose the complex) and the polysaccharide precipitated with acetone. It was then obtained in a dry condition (acid-free) in the normal way. The dry product was light brown in colour and this product was named fraction A. The insoluble copper-complex was similarly treated to regenerate the polysaccharide. The dry regenerated product was similar in appearance to the fraction A. This was named fraction B.

The fraction A comprised mainly of isolichenin and its purification process involved the removal of the accompanying polysaccharides which gave rise to mannose, galactose, etc., on hydrolysis and this was achieved by the method of "Fractional precipitation." The alkaline copper-complex solution of fraction A was treated with acetone gradually, when a slimy precipitate separated out. The addition of acetone was stopped at the point when there was no more slimy precipitate and it was removed from the solution by centrifuging. Further addition of acetone to the clear solution caused the separation of a flocculent precipitate which readily settled to the bottom. Addition of excess acetone was always avoided to prevent the precipitation of copper. The copper-

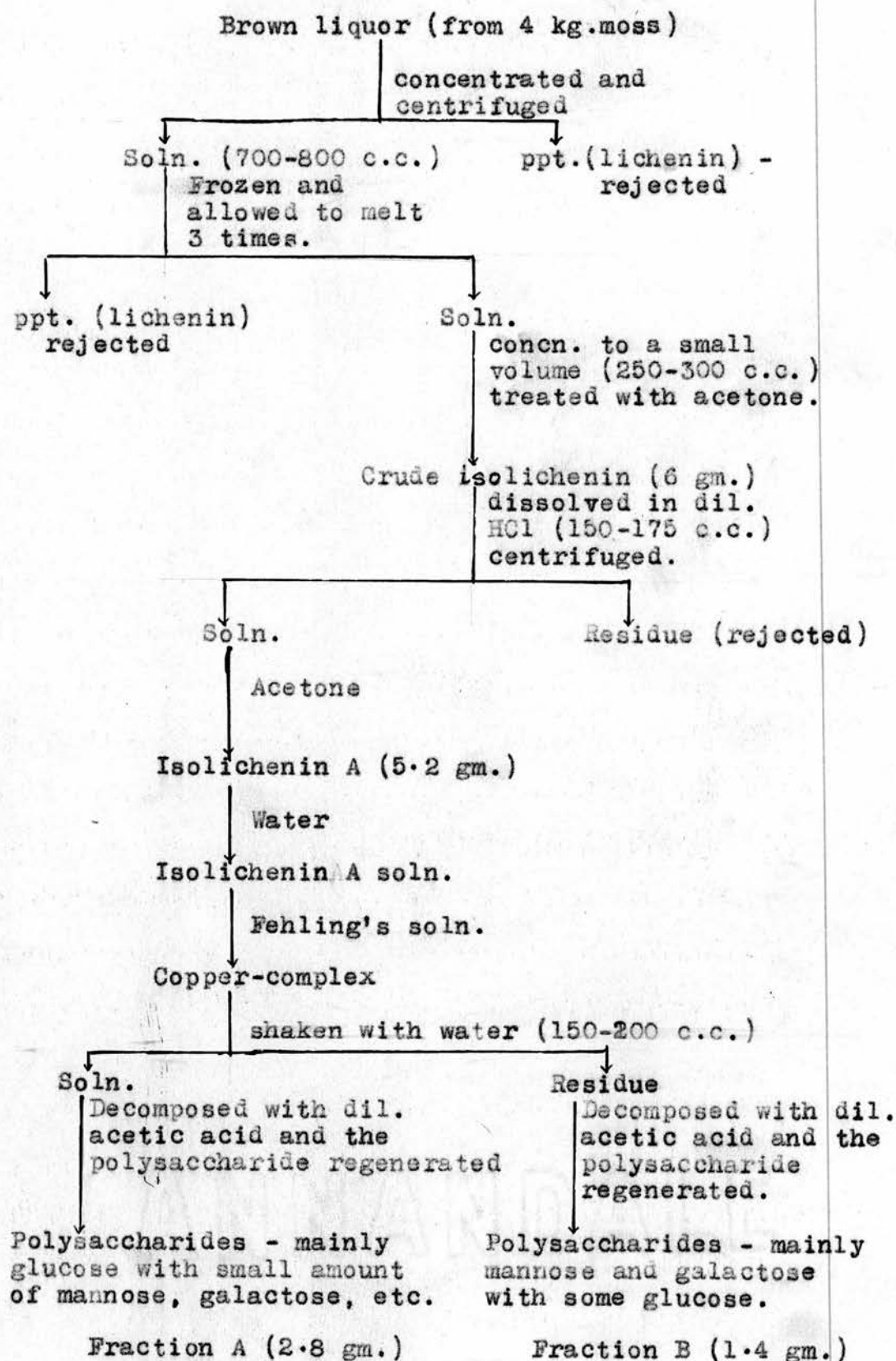
complex precipitate was then separated, dissolved in cold water by shaking, centrifuged and the clear solution was acidified with dilute acetic acid. By adding acetone to the acid solution, the polysaccharide was precipitated as a flocculent precipitate and obtained in a dry state (acid-free) in the way indicated previously. This was named isolichenin B. It should be noted here that the process of "incomplete precipitation" was adopted both in the precipitation of the copper-complex from the alkaline solution and the free polysaccharide from the acid solution.

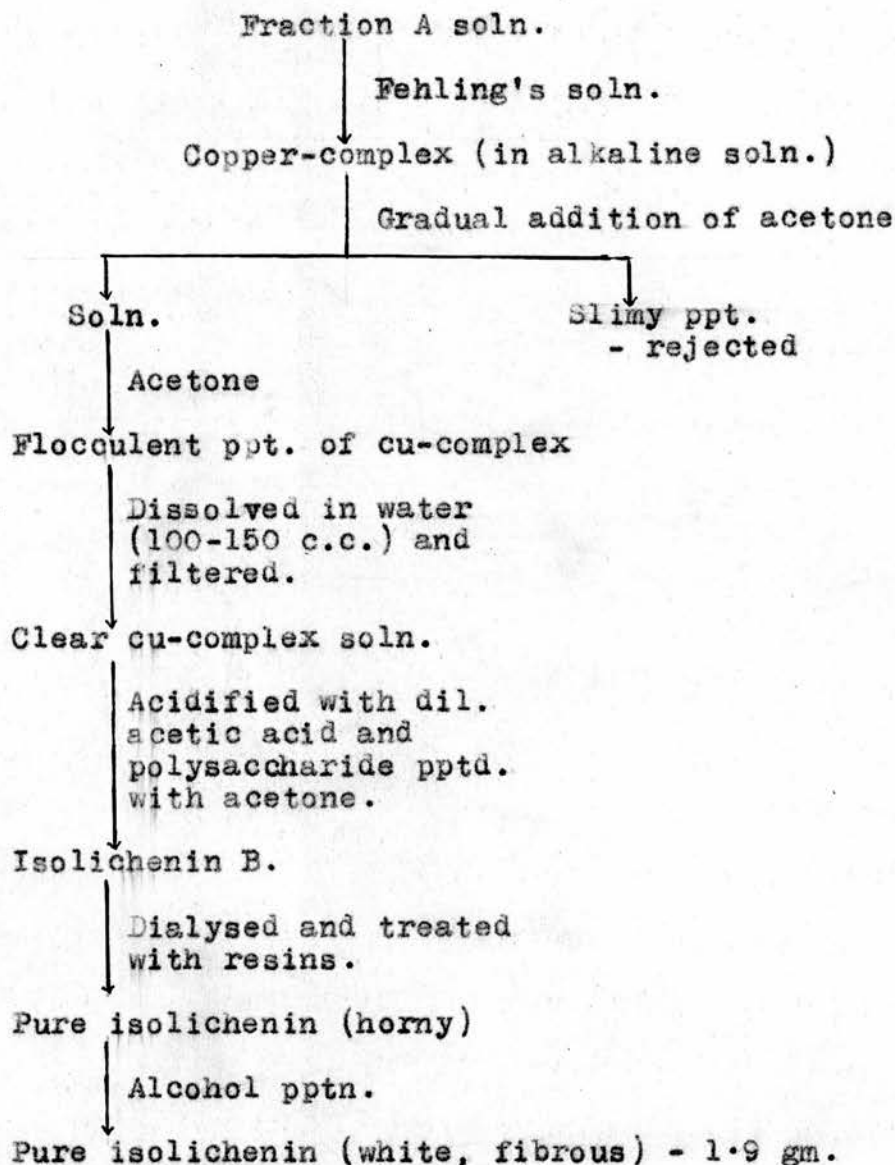
Chromatographic examination of isolichenin B, after hydrolysis, showed the presence of glucose only, proving thereby that the polysaccharide (isolichenin)^{was}/composed of glucose units only. The fractional precipitation removed all other polysaccharides (giving mannose, galactose, etc.) occurring with isolichenin.

The final purification process involved the removal of the degraded short-chain units and inorganic salts from isolichenin, this was achieved by dialysis for 2-3 days. The dialysed solution was concentrated in vacuo and passed in turn through a basic resin column and an acidic one to ensure complete removal of ions. The polysaccharide was then recovered from the solution as a hard horny solid.

In order to obtain the polysaccharide in a suitable form, it was dissolved in a minimum quantity of water by shaking for 2-3 hours. The solution was then centrifuged and the clear solution poured slowly into a large volume (1.5 l.) of absolute alcohol with stirring when the isolichenin precipitated as a white fibrous solid. The pure fibrous product was recovered by centrifugation, washed several times with absolute alcohol and then with ether and dried over phosphorus pentoxide in a vacuum desiccator. The pure product was hygroscopic to some extent and had an ash content of 0.32%.

The purification process for isolichenin is described above in a general way. The whole procedure is summarized in the chart below, the various quantitative figures mentioned are approximate and apply to the first batch of extraction.





In a second extraction, about, 0.8 gm. of pure isolichenin was obtained from about 2 kg. of moss.

The two samples of isolichenin, however, differed little in properties, having practically the same value for η_{sp}/c (in water) and also the same specific rotation in aqueous solution.

	n_{sp}/c	$[\alpha]_D^{15^\circ}$ in H_2O
Sample I	0.077	255.4°
Sample II	0.072	254.2°

Hydrolysis and quantitative estimation of glucose.

Isolichenin (10.32 mg.) was hydrolysed with 0.5N hydrochloric acid (1 c.c.) in a sealed tube for 3-4 hours and the glucose of the hydrolysed solution was estimated by the method of quantitative paper chromatography (Hirst, Jones, J.C.S., 1679, 1948), using ribose as a reference sugar.

Glucose content found: 95.67% of the theoretical.
No other sugar was detected on the paper chromatogram.

Blue-value of isolichenin.

Isolichenin gives a stable greenish-blue colour with iodine and in this respect, the polysaccharide resembles amylose. An attempt was made to measure the intensity of the greenish-blue colour as "Blue-value," which is commonly used in starch chemistry to determine the relative proportions of amylose and amylopectin. It has been found that the Blue-value of pure amyloses from various starches varies between 1.26 and 1.48, whereas the amylopectins have blue-values ranging from 0.06 to 0.16.

In determining the blue-value of isolichenin, the modified method of Bourne, Haworth, Macey and Peat, (J.C.S., 924, 1948) was used and the experimental

conditions are described below.

The dry isolichenin (10 mg.) was treated with water (1 c.c.) in a small test-tube and then with 2 drops of 2N-caustic soda solution. A blank was also run concurrently without isolichenin. After $\frac{1}{2}$ - 1 hour, the contents of each tube were washed into a 10 c.c. standard flask and diluted to the mark. A 5 c.c. portion of this solution (5 mg. isolichenin) was transferred into a 500 c.c. standard flask, the volume being made up to 100 c.c. with distilled water. Six drops of hydrochloric acid (3N) were added, followed by 5 c.c. of iodine solution (0.2% I_2 in 2% KI soln.). The solutions were then diluted to the mark and examined in the "Spekker" photoelectric absorptiometer using 4 cm. cells and red 608 filters.

The blue value of isolichenin was found to be 0.01, a figure much below that obtained for amylopectin.

Hypoiodite oxidation of isolichenin.

The oxidation was carried out as described in the case of lichenin: 59.39 mg. of isolichenin was found to consume 0.21 c.c. of 0.1028N I_2 solution, which corresponds to a chain length of 34 glucose units.

Periodate oxidation of isolichenin.

The principle and the experimental procedure involved in the periodate oxidation to determine the chain length of a polysaccharide was fully discussed

Moles formic acid / $C_6H_5O_5 \times 10^2 \times 2$

20
10
0

48

96

144

192

Time in hours →

POLTON

in the case of lichenin. The water-solubility of isolichenin permitted the carrying out of the oxidation in one batch, 99.61 mg. of isolichenin was dissolved in 70 c.c. water and treated with 0.4 gm of potassium periodate. A blank experiment was also carried out. 10 c.c. portion of the solution was titrated at fixed intervals. The number of moles of formic acid liberated per gm. mole of anhydroglucose was found to be as follows:

Time in hours	Moles formic acid/ $C_6H_{10}O_5$ $\times 10^2$
24	3.81
48	4.72
96	5.68
144	6.11
168	6.72
192	6.93

The amount of formic acid liberated was practically constant in the period 144 to 192 hours. An average of the result in this period gives a yield of 0.0658 moles of formic acid per anhydroglucose unit.

Assuming isolichenin to be a straight chain polymer of glucose, 3 moles of formic acid will be given by each molecule of the polysaccharide. According to this consideration, the amount of formic acid obtained experimentally corresponds to a chain length of about 45 units.

Periodate uptake of isolichenin.

115.41 mg. of isolichenin was dissolved in 50 c.c. of M/4 sodium metaperiodate solution and the oxidation was allowed to proceed in the dark. A blank experiment was also carried out simultaneously. The amount of periodate consumed by isolichenin was then estimated at fixed intervals by the method described in the case of lichenin. The results are shown below:

Time in hours	Moles of periodate consumed / $C_6H_{10}O_5$.
24	0.395
48	0.405
72	0.413
96	0.421

Isolichenin consumed only 0.4 mole of periodic acid per anhydroglucose unit which means that about 60% of the glycosidic linkages are of the 1:3-type and the rest may be 1:4-, 1:2-, 1:6-type or a mixture of the three.

Isolichenin Acetate.

Isolichenin acetate was prepared by the method described for lichenin acetate. In this case also the polysaccharide was thoroughly dispersed beforehand by means of pyridine and water (Pacsu and Mullen, J.A.C.S., 1487, 1941). From 1.8 gm. of isolichenin, 3 gm. of pure isolichenin acetate was obtained and this acetate was subsequently used for the preparation of methylated isolichenin. Acetyl content: 43.8%.

The product was white and fibrous in appearance and found to be soluble in pyridine, chloroform, acetone, etc. The following rotations were observed with different solvents:

In chloroform, $[\alpha]_D^{16}$, +160.4° (c, 1.035).

In acetone, $[\alpha]_D^{16}$, +156.2° (c, 1.031).

Viscosity of isolichenin acetate in chloroform.

Average time of flow	[solvent = 60 secs. solution = 74 secs.
at 20°C.	

when c = concn. in gm. per litre = 10.35 gm.

Therefore, $\eta_{sp}/c = 0.02254$.

Hence, D.P. = 43 units, when $K_m = 5.3 \times 10^{-4}$ in CHCl_3 .

(Staudinger and Reinedke, Ann., 535, 95,
1938).

Methylated isolichenin

Isolichenin was methylated via the acetate by the action of dimethyl sulphate and sodium hydroxide solution (30%), involving the action of simultaneous deacetylation and methylation. Through acetylation, the hydroxyl groups become "activated" and thereby favours the methylation reaction.

The acetate (2.7 gm.) in acetone solution was treated with dimethyl sulphate and sodium hydroxide solution (30%) dropwise from two dropping funnels, the average rate of addition being in the ratio of 1:3 drops. After the addition of 250 c.c. of dimethyl sulphate, the mixture was neutralized with dilute acetic acid and then dialysed for 4-5 days to remove inorganic salts. After concentrating the dialysed solution in vacuum, the partially methylated isolichenin was regenerated and subjected to further action of dimethyl sulphate and sodium hydroxide solution. At the latter stage of methylation, acetone was added to the reaction mixture to keep the partially methylated product in solution and the regeneration process was carried out by extraction with chloroform. Three methylations at room temperature and a fourth one at 30°C., using a total volume of 1000 c.c. of dimethyl sulphate, gave a product of methoxyl content 39.78%, soluble only in chloroform. For further methylation, Purdie's method was resorted to and three

separate treatments with methyl iodide (175 c.c.) and silver oxide raised the methoxyl content to 43.3%. At this stage, the methylated isolichenin (OMe, 43.3%) was fractionated by the solution method, using a mixture of pure petroleum (65°-70° B.P.) and chloroform. The different fractions obtained are indicated below:

Fraction	Solvent Petrol:CHCl ₃	Volume of solvent	Wt. of the fraction	Methoxyl content.
1	95:5	150 c.c.	-	-
2	90:10	200 c.c.	0.056 gm.	-
3	85:15	200 c.c.	1.398 gm.	43.6%
4	80:20	50 c.c.	0.661 gm.	42.2%

Fractions 3 and 4 were then combined and subjected to the liquid ammonia method to raise further its methoxyl content. It has been known for some time that some alcohols will react with sodium or potassium in liquid ammonia solution to form salts. Kraus, White and their collaborators (Kraus and White, J.A.C.S., 45, 768, 1923; White, *ibid.*, 45, 779, 1923; White and Knight, *ibid.*, 45, 1780, 1923; Kraus and Kawamura, *ibid.*, 45, 2756, 1923; etc.) have treated these salts in liquid ammonia with alkyl halides and thus formed the corresponding ethers. This observation was also found true and quite effective with sugars and polysaccharides. Muskat (J.A.C.S., 56, 693, 2449, 1934) and Freudenberg have developed this

method in the carbohydrate field. One important point is the solubility of the substance in liquid ammonia and it was found that all the ordinary sugars, their methylated, acetylated and acetone derivatives and even the polysaccharides are soluble in liquid ammonia. In practice, the substance is first dissolved in liquid ammonia and the calculated amount of sodium or potassium is added in small quantities. The course of the reaction may be followed by the blue colour, which the alkali metal in solution imparts to the ammonia, the loss of blue colour indicates that the alkali metal has reacted completely. In]this way, it is possible to titrate the sugar with sodium or potassium. The alkali salts are then treated with alkyl halide, using 15-20% excess over the theoretical amount.

In the present case, the methylation was carried out according to the method of Freudenberg and Boppel (Ber., 41, 2504, 1938). The reaction was performed in a tall wide glass tube with its lower end sealed. The top of the reaction vessel was fitted with a cork, carrying a mechanical stirrer. The complete arrangement was held in a Dewar flask, the annular space being filled with an acetone/solid CO₂ paste (-80°C).

2 gm. of the methylated isolichenin (fractions 3 and 4) was dissolved in about 70-80 c.c. liquid NH₃

contained in the reaction vessel. To ensure a complete solution, the mixture was stirred for 2-3 hours before adding sodium. Pieces of sodium (0.5 gm.) were then added to the solution and stirring continued for another 3 hours, when 2 c.c. of methyl iodide were slowly added to the solution. The stirring was continued for a further period of 3 hours, before the next additions. Further additions were as follows:

0.5 gm. sodium $\frac{3 \text{ hrs.}}{\text{stirring}}$ 2 cc. CH₃I added $\frac{3 \text{ hrs.}}{\text{stirring}}$ 0.5 gm. sodium
sodium

added $\frac{3 \text{ hrs.}}{\text{stirring}}$ 2 cc. CH₃I added and finally stirred for another 2-3 hours.

At the end of the reaction, the ammonia was allowed to evaporate and then neutralized with dilute acetic acid. The neutral mixture was extracted with chloroform, and the chloroform solution of the methylated isolichenin filtered, dried (over anhydrous Na_2SO_4) and concentrated in vacuum to a small volume. By pouring the concentrated solution into petrol ether (B.P. 40-60°), the methylated isolichenin was obtained as a white flocculent precipitate with a brownish tinge. The precipitate was then recovered and dried. The methoxyl content of the product was found to be 44.3%. Ash: 0.5%.

Fractionation of the methylated isolichenin.

The methylated isolichenin (OMe, 44.3%) was fractionated by the "solution" method and the properties of the different fractions are indicated below:

Fraction No.	Petrol:CHCl ₃ (100 c.c.)	Wt. of the fraction	η_{sp}/c in CHCl ₃	$[\alpha]_D^{14}$ in CHCl ₃	CH ₃ O-content
1	90:10	0.036 gm.	-	-	-
2	85:15	1.42 gm.	0.01373	217.6 (c=1.11)	44.52%
3	80:20	0.34 gm.	0.01837	216.2 (c=1.01)	44.12%

The above results indicated that the fractions 2 and 3 are almost identical and hence they were combined for hydrolysis. Before proceeding to hydrolyse the whole quantity, a test sample (10 mg.) was hydrolysed and examined chromatographically. The dimethyl glucose appeared on the paper as a very faint spot, which goes to indicate the completeness of methylation of the isolichenin. De-methylation during hydrolysis possibly accounts for this dimethyl glucose.

Viscosity of methylated isolichenin.

The viscosity of methylated isolichenin (MeO = 44.4%) in m-cresol was determined in an ordinary Ostwald viscometer. The result is shown below:

Average time of flow	[solvent (10 cc) = 482 sec.
at 20°C.	[solution (10 cc) = 615 sec.
	(c = 7.166 gm. per litre).

Therefore, $\eta_{sp}/c = 0.03851$

Hence, D.P. = 32 units, when $K_m = 12 \times 10^{-4}$

The methylated isolichenin had $[\alpha]_D^{15} +184.2^\circ$ in m-cresol (c, 0.71).

Hydrolysis of the methylated isolichenin.

The hydrolysis of the methylated isolichenin (1.4 gm.) was effected exactly in the way described in the case of lichenin, with one difference in that diazomethane was used to neutralize the acid

solution containing the glycosides. 1.4 gm. of the methylated isolichenin gave a partly crystallised syrup (slightly brown) weighing 1.5 gm. (partially dry). The syrup was a mixture of tetra-, tri-, di-methyl glucoses, possibly contaminated with some glycosides and a separation was carried out on a cellulose column (Hough, Jones, Wadman, Nature, 162, 448).

Separation of the different methyl glucoses.

A column of powdered cellulose (70 cm. x 2 cm.) was used in this separation. The solvent employed was a water saturated mixture of 60% purified petrol ether (B.P. 100-120°) and 40% purified n-butanol. The experimental procedure followed was exactly the same as indicated in the case of lichenin.

The quantities of the different sugars obtained by the above separation were as follows:

- | | |
|---|------------------|
| 1. Tetramethyl glucose fraction : | 0.161 gm. |
| 2. Trimethyl glucose fraction : | 1.205 gm. |
| (Mixture of 2:3:6- and 2:4:6-trimethyl glucose) | |
| 3. Dimethyl glucose fraction : | 0.040 gm. |
| 4. "Water-washed" fraction: | <u>0.013 gm.</u> |

Total: 1.419 gm.

The high weight of the end-group fraction together with its failure to crystallise even on long standing indicated the presence of some glycosides and this was confirmed by hydrolysing a test sample of this fraction

when two spots corresponding to the tetramethyl and trimethyl glucoses were obtained. The trimethyl fraction showed two spots (75 cm. paper; 50 hours run), one yellowish brown spot corresponding to the 2:3:6-trimethyl glucose and the other, a reddish pink spot corresponding to the 2:4:6-trimethyl glucose. This fact of the presence of the two trimethyl glucoses together with the relative intensities of the two spots readily agreed with the result of the periodate uptake of isolichenin. On running a paper (75 cm.) for 2 days, the dimethyl fraction showed the presence of 2:3-dimethyl glucose and a second spot just touching the 2:3-spot, representing a mixture of other dimethyl glucoses, possibly 2:6-, 3:6-dimethyl glucoses etc., which do not separate.

The "water-washed" fraction was found to contain traces of monomethyl glucose and glucose itself, these possibly being produced from the other sugars by demethylation.

Purity of the different fractions.

The purity was ascertained by hypoiodite oxidation and the results are shown below:

Tetramethyl glucose fraction : 18.0%

Trimethyl glucose fraction : 90.3%

Dimethyl glucose fraction : 70%

The amounts of pure sugars obtained, calculated

on the basis of the above per cent of purity are as follows:

2:3:4:6-Tetramethyl glucose	:	0.031 gm.
Trimethyl glucose	:	1.220 gm.
Dimethyl glucose	:	0.028 gm.

Total 1.279 gm.

Per.cent. of total recovery = 91.3%

Assuming isolichenin to be a straight chain polymer, the end-group assay shows the chain length to be 53 units.

Rehydrolysis and separation of the end-group fraction.

The end-group fraction (161 mg.), representing a mixture of tetramethyl glucose and trimethyl-methylglycoside, was rehydrolysed with 0.5N hydrochloric acid (10 c.c.) and the sugars separated on a column. The following amounts of the two sugars were obtained:

2:3:4:6-Tetramethyl glucose	:	19 mg.
Trimethyl glucose (Mixture of 2 trimethyl glucoses).	:	118 mg.

Examination of the trimethyl fraction.

The trimethyl fraction (1.205 gm.), examined chromatographically, was a mixture of 2:4:6- and 2:3:6-trimethyl glucoses, a fact agreeing with the periodate uptake result of isolichenin. The absence of both 3:4:6-trimethyl glucose and 2:3:4-trimethyl glucose in this fraction was proved by negative Weerman and

chromatographic tests. These facts indicated the presence of only 1:3 and 1:4 linkages in the iso-lichenin molecule.

Separation of 2:4:6- and 2:3:6-trimethyl glucoses.

It may be mentioned here that some pure 2:4:6-trimethyl glucose (100 mg.) was obtained during the separation of the original mixture of methylated glucoses. In a subsequent operation, 150 mg. of pure 2:4:6-trimethyl glucose and 50 mg. of pure 2:3:6-trimethyl glucose were obtained from 300 mg. of the mixture by repeated separation (3 times) on a cellulose column.

Dimethyl glucose fraction.

The dimethyl fraction (40 mg.) was found by the hypiodite oxidation to be 70% pure, i.e. the fraction contained 28 mg. pure sugar. It was a syrup and a qualitative examination on a long chromatogram (75 cm., 50 hours) showed the presence of 2:3-dimethyl glucose together with ^afew other dimethyl glucoses, possibly 2:6-, 3:6-dimethyl glucoses etc., which do not separate. This difficulty together with the small amount of the fraction did not permit a thorough examination of the different dimethyl glucoses. The relative proportions of 2:3-dimethyl glucose and other dimethyls (2:6-, 3:6-dimethyl glucoses etc.) was approximately determined by a quantitative paper

chromatography (Hirst, Jones and Flood, J.C.S., 1679, 1948). For this purpose, the impure sample was purified by treatment with charcoal and subsequent acetone extraction and then analysed in the usual way. The analysis indicated the presence of 20.7% (5.8 mg.) 2:3-dimethyl glucose and 79.3% (22.2 mg.) other dimethyl glucoses in the mixture.

Characterization of the Sugars.

1. 2:3:4:6-Tetramethyl glucose was purified by crystallizing three times from pure dry light petrol (B.P. 40-60°) and the pure sample had m.p. 83°-85°C.; $[\alpha]_D^{17}$, 83.8 at equilibrium (c, 0.53 in water).

2. 2:4:6-Trimethyl glucose was purified by recrystallization from dry ether and had m.p. 120-122°C., $[\alpha]_D^{18}$, 75.2 at equilibrium (c, 1.1 in water); OMe, 41.06%; C, 49.4%; H, 8.0%. ($C_9H_{18}O_6$ requires OMe, 41.9%; C, 49.1%; H, 8.1%).

48.6

2:4:6-Trimethyl glucose anilide.

The anilide was made by the method previously described (p. 73). It had m.p. 143-145°C.; C, 60.43%; H, 7.6%. ($C_{14}H_{23}O_5N$ requires C, 60.2%; H, 7.7%; N, 4.7%).

The nitrogen content could not be determined for want of material.

An anilide made from an authentic sample of 2:4:6-trimethyl glucose had m.p. 142-144°C.

3. 2:3:6-Trimethyl glucose was purified by recrystallization from pure dry ether and had m.p. 114-116°C; $[\alpha]_D^{17}$, 67.3 at equilibrium (c, 0.58 in water), C, 47.7%; H, 6.97%. ($C_9H_{18}O_6$ requires C, 49.1%; H, 8.1%).

Phenylhydrazide of 2:3:6-trimethyl glucose, was prepared by the previously described method (p. 72) It had m.p. 145°; OMe, 28.9%. ($C_{15}H_{24}O_6N_2$ requires 28.4%).

There was not enough material for further analysis.

Action of Beta-amylase on Isolichenin.

The high positive rotation ($[\alpha]_D^{15}, 254^\circ$) of isolichenin indicates the presence of α -glucosidic links in the molecule and it is to be expected that it would be attacked by β -amylase, if (a) the isolichenin preparation were a mixture of two different polysaccharides with 1:4- and 1:3-linkages, or (b) the polysaccharide contained both the linkages in a suitable pattern favourable to the action of β -amylase. The action of β -amylase, either positive or negative, will no doubt provide some information as to the nature (homogeneous or heterogeneous) of the isolichenin preparation.

Pringsheim and his co-workers (Ber., 57, 1594, 1924) studied the action of diastase (Kahlbaum) on isolichenin and claimed a 100% conversion of isolichenin into maltose by measuring the reducing power and the rotation of the hydrolysed solution. By the action of emulsin, the above authors also claimed a 49% conversion into maltose. In the great controversy about the nature of isolichenin with Karrer (see Introduction), Pringsheim used the above results as the basis of his claim that isolichenin was nothing but an amylose.

As one approach to this problem, studies have been made of the action of pure β -amylase (free from α -amylase) on isolichenin instead of diastase which is

no doubt a mixture of several enzymes. In view of the heterogeneous character of diastase, the results of Pringsheim's investigation are hard to evaluate in terms of the action of a single enzyme. Beta-amylases, when used as a tool to elucidate the structure of their substrates, must be free from other enzymes and from other naturally associated constituents which may influence the results. Beta-amylases from several sources have been prepared by selective inactivation of other enzymes that accompany them in nature and highly active products have been obtained by extensive purification. Ball and his associates (Ball, Thompson and Walden, J.Biol.Chem., 163, 571, 1946; 173, 9, 1948) have recently reported the crystallization of beta-amylase from sweet potatoes.

Preparation of Beta-amylase from Barley.

Beta-amylase was prepared from barley according to the method of Preece (Preece and Shadaksharaswamy, Biochem.J., 44, 271, 1949), which is actually a modification of the method of Hopkins, Murray and Lockwood (Biochem.J., 40, 507, 1946). In practice, the ground barley was extracted with 2.5 times its weight of 20% (v/v) alcohol for 30 minutes with stirring, the mixture filtered and the concentration of alcohol adjusted to 50% (v/v). After allowing to stand for 24 hours, the precipitate was filtered off and discarded, and the concentration of alcohol

in the filtrate adjusted to 80% (v/v). The precipitate (β -amylase) was then collected by centrifuging and washed several times with absolute alcohol and dried in the usual way. This preparation, according to Preece, contained only sufficient α -amylase to produce 1/750 of the saccharification due to beta-amylase.

Action of β -amylase on starch.

The activity of the β -amylase, prepared by the method described above, was first tested using starch as a substrate. A.R. soluble starch was used in the experiment, the details of which are given below:

2 c.c. 1% starch solution] Incubating temp., 37°-39°C. Time, 24 hrs.
0.2 c.c., 0.1% β -amylase soln.	
2 c.c. acetate buffer (pH	
4.6) per 100 c.c. starch	
solution.	
1 drop toluene.	

At the end of the incubating period, salts are removed from the solution by treatment with Amberlite resins. Paper chromatographic analysis of this solution in pyridine solvent (benzene 1, pyridine 3, n-butanol 5, water 3) gave a spot corresponding to maltose and no glucose was detected which indicates the absence of α -amylase in the enzyme preparation. In the case of starch, maltose can be detected in the solution even after 15 minutes incubation. A blank experiment without the starch was also carried out to discover if the enzyme itself gave any spot on the chromatogram but none could be detected.

MLY
glucose
maltose starch

iso

Isolichenin

Maltose

glucose

Photograph of the Chromatogram illustrating the inactivity of β -amylase towards isolichenin.

Action of β -amylase on Isolichenin.

Having tested the activity of the β -amylase preparation, isolichenin was subjected to its action under exactly the same conditions under which it attacks starch. The details of the experiment are given below:

50 mg. isolichenin in 5 cc. water (1% solution)	Incubating
* 0.5 cc. 0.1% β -amylase solution	temp., 37°-39°C.
2 cc. acetate buffer (pH = 4.6)	Time, 48 hrs.
per 100 cc. isolichenin soln.	
1 drop toluene	

* In 98 c.c. water, 2 c.c. buffer was added and 5 c.c. of this mixture used to prepare the isolichenin solution.

After 48 hours the solution was treated with resins (acidic and basic) and then taken to dryness in vacuo. The solid was extracted with 85% hot alcohol (by refluxing), which will no doubt dissolve all free sugars if any were present. The extract was then concentrated and examined chromatographically but no sugar was detected. In the control experiment with starch, maltose was identified. The attached photograph of the chromatogram illustrates the above result. A number of duplicate experiments were carried out with isolichenin but in all cases, the result was negative. The solution was also found to give the same intensity of colour (greenish-blue) with iodine as was given by a fresh solution of isolichenin of the

same concentration, this fact also proves that the isolichenin is inert towards β -amylase.

Action of soyabean β -amylase on starch and isolichenin.

The action of soyabean β -amylase on starch and isolichenin was also studied and in this case, also, its inactivity towards isolichenin was recorded.

A positive result was obtained with starch.

Soyabean β -amylase was kindly supplied by Professor S. Peat, the activity of the enzyme preparation being 8,600 units per c.c. (Hobson, Whelan and Peat, J.C.S., 3570, 1950). The action of soyabean β -amylase on starch and isolichenin was studied in exactly the same way and under the same conditions described in the case of barley β -amylase. The soyabean β -amylase was in ammonium chloride solution and one drop of this solution was used in each experiment.

Non-poisonous effect of isolichenin.

The negative result of the β -amylase action on isolichenin may be due to the poisoning of the enzyme by the polysaccharide itself. In order to clear up this point, an experiment was carried out with a solution which contained 50% starch and 50% isolichenin, the details of which are given below;

2 cc 1% soln., containing 10 mg
isolichenin and 10 mg. starch
0.2 cc., 0.1% β -amylase soln.
2 cc acetate buffer (pH=4.6)
per 100 cc. substrate soln.
1 drop toluene.

Incubating temp.,
37° - 39°C.

Time, 24 hrs.

Maltose was detected chromatographically in the solution, which goes to prove that the polysaccharide itself did not exert any poisoning effect on the enzyme.

In all the experiments, isolichenin was found to be resistant to the action of β -amylase, a significant result to be discussed later on.

Discussion.

The isolichenin, a glucosan, was obtained from Iceland moss (*Cetraria islandica*) where it occurs in the cell-wall in very small quantity, as shown by the fact that 2.7 gm. of the polysaccharide was obtained from about 6 kg. of moss. During hot water extraction the polysaccharide comes out along with lichenin and its presence can be detected by the characteristic greenish-blue colour it acquires with dilute iodine solution.

The first attempt to elucidate the chemical nature of isolichenin came from Pringsheim and Karrer independently and in fact, they obtained quite contradictory results. Pringsheim claimed that isolichenin was a pure glucose polymer resembling amylose, whilst Karrer and his co-workers claimed that isolichenin was a mixture of several polysaccharides, giving glucose, mannose, and galactose on hydrolysis. Recently, Meyer, et.al. (Helv., 30(1), 761, 1947) made an attempt to discover the real chemical nature of isolichenin with no success. By fractional precipitation of the crude isolichenin, they obtained five fractions (see Introduction) of different properties and from this study, they concluded that isolichenin was a mixture of several polysaccharides, a result similar to that of Karrer.

It is quite evident that the first essential in

the study of isolichenin is the isolation of the substance in a pure state. In the present investigation, a purification process has been developed (p. 107) which gave a pure specimen of isolichenin - pure in the sense that it did not give any other sugar, on hydrolysis, except glucose. All the associated polysaccharides giving mannose, galactose, etc. on hydrolysis have been removed during the various purification stages. There still remains a question to settle whether the pure isolichenin preparation is homogeneous in character or a mixture of different polyglucosans - the true answer to this question is rather difficult and an attempt will be made in this discussion to answer this question in the light of some experimental results obtained from an enzymatic study.

Isolichenin was found to be a glucose polymer possessing some characteristic properties not very common in polysaccharide chemistry, such as high solubility in cold water, high positive optical rotation and characteristic greenish-blue colour with dilute iodine solution. The glucose content was found to be about 96% and this discrepancy of 4% may possibly be due to the destruction of the sugar during acid hydrolysis of the polysaccharide. No uronic acid could be detected in the pure sample either by paper chromatogram or by the naphthoresorcinol test.

Isolichenin possesses a very high dextro-rotatory power in aqueous solution: $[\alpha]_D^{15}$, $+254^\circ$ (c, 1) and this rotation of isolichenin is the highest so far recorded for any polysaccharide, cf. starch, $[\alpha]_D = +190$ and glycogen, $[\alpha]_D = +192$.

This high positive rotation of isolichenin points without any doubt to the presence of α -glycosidic links in the molecule. Another characteristic property of isolichenin is the greenish-blue colour with dilute iodine solution, a property indicating some structural relationship with amylose. A few colour reactions of some polysaccharides with iodine are recorded below:

Amylose	: blue
Amylopectin	: reddish violet
Glycogen	: brownish red
Floridean starch	: reddish brown to violet
Glucosan from Lycoperdon bovista	: reddish brown.

The greenish-blue colour of isolichenin with iodine is stable and serves as a test for its detection. This characteristic colour with iodine together with its high positive rotation led Pringsheim to believe that isolichenin is nothing but amylose. An attempt was made to measure this colour intensity as "Blue-value" of a starch-iodine complex, according to the method of Bourne, Haworth, et.al. (J.C.S., 924, 1948). The blue-value was found to be 0.01, a value far below

that of amylose whose value lies in the range 1.26 to 1.48, depending on the nature of ^{the} starch.

To elucidate the constitution of isolichenin, the polysaccharide was methylated by means of dimethyl sulphate and caustic soda. Three methylations (Haworth's method) at room temperature and a fourth one at 30°C., using a total volume of 1000 c.c. dimethyl sulphate, gave a product of methoxyl content 39.78%, soluble in chloroform. This product was then subjected to three separate treatments with Purdie's reagents which raised the methoxyl content to 43.3%. At this stage, the methylated product (MeO = 43.3%) was fractionated by the solution method, using a mixture of pure petroleum ether (65-70°) and chloroform. After examining the different fractions, the fractions 3 (OMe, 43.6%) and 4 (OMe, 42.2%) were combined and subjected to further methylation by the liquid ammonia method. This treatment gave a product of 44.4% methoxyl content. On fractionation of this product, three fractions were obtained and on examination, the fractions 2 and 3 were found to be almost the same as shown below:

Fraction	Wt. in gm.	n_{sp}/c in $CHCl_3$	$[\alpha]_D^{14}$ in $CHCl_3$	OMe%
2	1.42	0.01378	217.6	44.52
3	0.34	0.01837	216.2	44.12

The above results indicated that the two fractions are similar and so they were combined for hydrolysis.

Before proceeding to hydrolyse the whole amount, a test sample (20 mg.) was hydrolysed and examined chromatographically. Dimethyl glucose appeared on the chromatogram faintly, which proved the completion of methylation. Demethylation during hydrolysis possibly accounts for the dimethyl glucose obtained on the chromatogram.

The methylated isolichenin (1.4 gm.) was then hydrolysed to a mixture of methylated glucoses and the different sugars separated on a cellulose column in the normal way. The different sugars were obtained in the following amounts:

Tetramethyl glucose fraction (syrup): 0.161 gm.

Trimethyl glucose fraction : 1.205 gm.

Dimethyl glucose fraction : 0.040 gm.

Small amounts of monomethyl glucose and glucose itself were also obtained by washing the column with water at the end.

Hypoiodite oxidation of the end-group fraction (syrup) indicated the presence of 31 mg. (18%) of tetramethyl glucose which corresponds to one non-reducing end-group for every 53 units. The syrup was rehydrolysed and separated on a column when the sugars were obtained in a crystalline condition.

The trimethyl glucose fraction (1.205 gm.) was found to contain 2:4:6- and 2:3:6-trimethyl glucoses, a result in agreement with the observed periodate

uptake (0.4 mole) of isolichenin. The fraction (first oxidised with bromine^{to the} acid and then converted into the amide), gave a negative Weerman reaction showing the absence of 3:4:6-trimethyl glucose and also a negative chromatographic test for the 2:3:4-trimethyl glucose. These negative tests eliminated the possibility of the presence of 1:2- and 1:6-linkages in the polysaccharide.

The amount of dimethyl glucose is important with reference to the presence of branching in the isolichenin molecule. The dimethyl fraction (28 mg.) was a mixture of 2:3-dimethyl glucose and some other dimethyl glucoses, possibly 2:6-, 3:6-dimethyl glucoses, etc., which do not separate on a chromatogram. An approximate analysis showed the presence of 20.7% (5.8 mg.) 2:3-dimethyl glucose and 79.3% (22.2 mg.) other dimethyl glucoses in the mixture. Theoretically in a singly branched chain with one non-reducing end-group, the amount of the dimethyl sugar should be in equivalent proportion to the end-group. Now in a molecule of about 50 residues (end-group assay) size as isolichenin appears to be, the amount of dimethyl glucose derived from branching can only be 26 mg., a value in agreement with the amount of dimethyl glucoses (28 mg.) obtained and this consideration shows the presence of a branched chain. Again when the phenomenon of demethylation is considered, the amount of dimethyl glucoses is within the range of the demethylation

observed with pure specimens of 2:4:6- and 2:3:6-trimethyl glucoses (p.85-6) and this fact readily eliminates the question of branching of the isolichenin molecule.

The structural details suggested by the results of these methylation studies have been amplified by an examination of the action of periodate on isolichenin. The periodate uptake results showed the isolichenin molecule contained 60% 1:3-linkages and 40% 1:4-linkages and this fact was substantiated by the presence of both 2:4:6- and 2:3:6-trimethyl glucoses in the trimethyl glucose fraction. In this respect, isolichenin differs from ordinary amylose in which the linkage (1:4) is uniform but resembles Floridean starch (Hirst, Jones, Barry and Halsall, J.C.S., 1468, 1949) which has 40% 1:3-linkages. The presence of mixed linkages with a high proportion of the 1:3-type differentiates isolichenin (and also lichenin) from all other land-plant polyglucosans which are made up of 1:4-linkages. This type of linkage (1:3) is widespread in algal polysaccharides (Laminarin, Floridian starch, etc.) and constitutes a distinct difference between marine and land-plant polysaccharides in which the linkages are predominantly 1:4. The only other recorded glucan which possesses exclusively this unusual type of linkage (1:3) is that from yeast. Zechmeister, et.al. (Biochem.Z., 270,

309, 1934) and Hassid, et.al. (J.A.C.S., 63, 295, 1941) have examined this polysaccharide which furnishes only 2:4:6-trimethyl glucose on exhaustive methylation and subsequent hydrolysis.

During periodate oxidation, the amount of formic acid liberated was found to increase slightly with time at the end, the cause of which must probably be over-oxidation. By taking a mean value for the period 144 to 192 hours, 0.0666 mole of formic acid per $C_6H_{10}O_5$ residue was liberated. Assuming the chain to be linear, the above result corresponds to a chain length of 45 units. Hypiodite oxidation of isolichenin indicated one reducing group per 34 units while the viscosity determinations of methylated and acetylated isolichenin gave the following values for the D.P. of isolichenin.

Methylated isolichenin (in m-cresol) = 32 when
 $k_m = 12 \times 10^{-6}$

Isolichenin acetate (in chloroform) = 43, when
 $k_m = 5.3 \times 10^{-4}$

These values for D.P. are open to question in view of the various limitations of the viscosity equation.

From the above consideration of the various results obtained in the study of isolichenin, it seems probable that the polysaccharide is a linear polymer of glucose having a chain length of 40 ± 5 units.

It is hoped that when more accurate determinations of molecular weight are forthcoming a conclusive answer can be given.

Another important point yet to be discussed is the homogeneous character of the isolichenin preparation used in the present investigation. The periodate uptake result indicates the presence of about 60% 1:3-linkages in the molecule, the remaining 40% being 1:4-linkages and this result was substantiated by the methylation study. This fact of mixed linkages raises a point as to whether the isolichenin preparation is homogeneous, having the two types of linkages (1:3 and 1:4) in the same chain or whether the preparation is a mixture of two different glucans of 1:3 and 1:4 linkages. In an attempt to answer this question, the action of β -amylase on isolichenin was studied. The high positive rotation ($[\alpha]_D^{15}$, $+254^\circ$ in water) of isolichenin indicates the presence of α -glycosidic links in the molecule and it is to be expected that β -amylase would attack the 1:4-linked glucose residues giving maltose, a phenomenon similar to the splitting of starch and glycogen by β -amylase to maltose. With this object in view, the action of β -amylase on isolichenin was studied and in all cases the results were found to be negative. The stability of isolichenin towards β -amylase suggests that the preparation represents a single glucan of mixed linkages.

The β -amylase is believed to attach itself to free non-reducing end groups of the chains and attacks 1:4-linkages to give maltose units until the substrate deviates from the simple 1:4- α -glucopyranoside linkage pattern. From this consideration, the isolichenin molecule, since it is not attacked by β -amylase, does not have at the non-reducing end, the following arrangement:

(a) $G_1 - 4G_1 - 4G_1 - - -$

but would have either

(b) $G_1 - 4G_1 - 3G_1 - - -$

(c) $G_1 - 3G_1 - 3G_1 - - -$

or (d) $G_1 - 3G_1 - 4G_1 - - -$

where G = anhydroglucose unit.

It is not known whether β -amylase is able to split off laminaribiose but if this is the case then arrangement (d) is also eliminated. In the arrangement (c), the remaining 1:3-linked glucose residues may be in continuation of the first three residues (1:3) which form the non-reducing end of the chain and these are followed by the 1:4-linked residues, i.e.

$G_1 - 3G_1 - 3G_1 - 3G_1 - 3G_1 - 3G_1 - - 4G_1 - 4G_1 - 4G_1 - \dots (E)$

The above pictures of the non-reducing end of the molecule are suggested merely from a theoretical consideration of the observed inactivity of β -amylase towards isolichenin. In the case of the arrangement (E), α -amylase will probably liberate maltose because

the enzyme has the capacity of attacking and rupturing any maltose linkage in a chain molecule of the starch type and this point will be investigated in due course. Besides the study of the action of α -amylase, more experimental evidence particularly from a study of different oligosaccharides (from partial hydrolysis of isolichenin) is needed to form a correct picture of the isolichenin molecule.

Summary.

1. Isolichenin, a glucosan, was extracted from Iceland moss (*Cetraria islandica*) and the pure product gave only glucose on hydrolysis. The polysaccharide has a greenish-blue colour reaction with dilute iodine solution.
2. Isolichenin has $[\alpha]_D^{15}$, +254° (c, 1.06 in water) and this high positive rotation indicates the presence of α -glucosidic links in the molecule.
3. Periodate uptake of isolichenin showed that the polysaccharide possesses about 60% 1:3-linkages and the remaining portion is of the 1:4-type.
4. Exhaustive methylation and subsequent hydrolysis of isolichenin gave four methyl glucoses: 2:3:4:6-tetramethyl glucose, 2:4:6-trimethyl glucose, 2:3:6-trimethyl glucose, 2:3-dimethyl glucose and a few other dimethyl glucoses; possibly 2:6-, 3:6-dimethyl glucoses, which do not separate on a chromatogram. The amount of dimethyl glucoses was approximately 2%, which possibly originated from demethylation and some under-methylation.
5. The end-group assay indicated the chain length of isolichenin to be 53 units whilst the periodate oxidation result corresponded to a D.P. of about 45 units. Hypiodite oxidation of the polysaccharide gave^a much lower value (34) for the D.P.
6. Viscosity measurements on methylated isolichenin

showed the D.P. to be 32 units whilst the same method with isolichenin acetate in chloroform gave a value of 43 units.

7. *An* accurate molecular weight of the polysaccharide is needed to form a correct picture of the molecule. From the various results, it seems probable that isolichenin is a linear polymer of glucose having a chain length of 40 ± 5 units.

8. Isolichenin was found to be inert towards β -amylase and this inertness suggested the homogeneous character of the polysaccharide preparation. On the basis of the observed inactivity of β -amylase towards isolichenin, three possible structures of the non-reducing end of the molecule have been suggested.

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ANNANDALE

POLTON